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SYNTHETIC NUCLEOTIDES
AND THEIR BIOLOGICAL APPLICATIONS

A thesis submitted in partial fulfilment of the
requirements for the Degree of Doctor of Philosophy
at the University of Warwick.

by

John I. Ademola

August 1980

..... To My Family

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DECLARATION

The work described in this thesis is the original work of the author, except where acknowledgement is made to work and ideas previously published. It was carried out in the Department of Chemistry and Molecular Sciences and the Department of Biological Sciences, University of Warwick between 1977 and 1980, and it has not been submitted previously for a degree at any institution.

ABBREVIATIONS

2-5A 5'-O-triphosphoryl-adenylyl-(2'-5')-adenylyl-(2'-5')adenosine

Abs Absorbance

Ado Adenosine

br⁸ AMP 8-bromo adenosine monophosphate

BS- Benzene sulphonyl-

BHK-21 Baby hamster kidney cells-21

C.D. Circular dichroism

Core=trimer adenylyl-(2'-5')-adenylyl-(2'-5')-adenosine

DCC Dicyclohexylcarbodiimide

DEAE Diethylaminoethyl

(dIn₃)_n·(C)_n Base paired hybrid between polyribonucleotides, in which 2'-OH of the (I)_n strand is replaced by 2'-azido group

DMT-cl Dimethoxytrityl chloride

DNA Deoxyribonucleic acid

ds Double stranded

EDTA Ethylenediaminetetraacetic acid

EMCV Encephalomyocarditis virus

Guo Guanosine

HFF Human foreskin fibroblast

I.C. Base pairs

I_n·C_n Base paired hybrid between polyribonucleotides

IF Interferon

ImpA Adenosine 5'-phosphorimidazolid

k_i Inhibition constant

k_m Michaelis constant

MPDA	9(4'-methoxyphenyl)-10,10-dimethylantran-9-yl
MMT-cl	Monomethoxytrityl chloride
mRNA	Messenger ribonucleic acid
MS-	Mesitylene sulphonyl-
MW	Molecular weight
NDV	Newcastle disease virus
NDP	Nucleoside diphosphate
PBS	Phosphate-buffered saline
2'PDi	2'-phosphodiesterase
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SFV	Semliki Forest Virus
ss	Single stranded
SV40	Simian Virus 40
TCA	Trichloroacetic acid
t.l.c.	Thin layer chromatography
T _m	Melting temperature
TPS-	Triisopropyl sulphonyl-
Trityl-Cl	Triphenylmethyl chloride (Triphenylchloromethane)
VSV	Vesicular stomatitis virus
⁸ z AMP	8-azido adenosine monophosphate

SUMMARY

The chemical synthesis and characterisation of the nucleotides, 2-5A and 8-azidoadenosine-5'-monophosphate was accomplished. The activity of both nucleotides was tested in appropriate biological systems.

(1) Synthesis and biological properties of 2-5A.

(a) The interferon messenger, pppA2'p5'A2'p5'A(2-5A) was synthesised in three stages. First, the 'core' A2'p5'A2'p5'A was chemically synthesised by Khorana's diester method. The core was then monophosphorylated first by wheat shoot phosphotransferase in the presence of ATP. This was followed by chemical phosphorylations, to yield 2-5A with a terminal triphosphate.

(b) During the synthesis of 2-5A, the rates of acid hydrolysis of nucleoside and nucleotide protecting groups were studied. Substituted trityl- and isopropylidene adenosine derivatives were found to have optimum properties for use during oligonucleotide synthesis. The efficiencies of various condensing agents were investigated and it was concluded that for the phosphodiester method, the use of sulphonyl imidazoles offered the best method for linking together alcohol function of nucleotides and nucleoside phosphomonoesters.

The synthetic 2-5A and related nucleotides were characterised by both chemical and enzymatic methods. In preliminary studies, 2-5A at mM concentrations was shown to

inhibit protein synthesis in permeabilised MG63 cells, the replication of Semliki Forest virus in permeabilised MG63 cells, and the growth of MG63 and HFF cells. In one experiment 1.4M 2-5A produced 50% inhibition of protein synthesis, but with other preparations of permeabilised cells much higher concentrations were required. It was also observed that MG63 cells yielded high amounts of interferon following incubation with poly I.C.

- (2) The properties of wheat shoot phosphotransferase were investigated, following its purification by several steps including affinity chromatography on Matrex Gel Blue A. The use of the enzyme in nucleotide synthesis was investigated. The enzyme was immobilised on various insoluble supports and these forms of the enzyme were also used for the synthesis of nucleotides. Benzoquinone-immobilised phosphotransferase attached to Sepharose gave the highest yield of nucleotides under the conditions investigated.
- (3) Finally, in the final chapter of the thesis, a study was made of the interaction between a photoactivated affinity labelling reagent, 8-azidoadenosine-5'-monophosphate and the enzyme staphylococcal nuclease. The kinetics of the inhibition of the enzyme by the reagent were studied and were found to be competitive with substrate treatment. Following cleavage of the enzyme into five peptides by cyanogen bromide treatment, it was observed that 80% of the radioactive photolabel was recovered in one peptide, while a second peptide contained the remaining 20%.

This later study was undertaken as a model for the type of work that would be required to identify the biological receptors for 2-5A in the cell. However, one receptor, endonuclease, which cleaves mRNA, is already known.

CHAPTER ONE

Interferons are cellular glycoproteins able to induce broad spectrum, species-specific antiviral activity in sensitive cells, through processes requiring new cellular RNA and protein synthesis. The relative sensitivities are determined by a host-range of cells. Apart from their antiviral activity, interferons exhibit various other alterations in the cells, including priming, blocking and enhancement to toxicity of dsRNA. The effect of priming on the actions of interferons was first observed by Burke and Isaacs (1958), who found that treatment of cells with small amounts of interferon prior to addition of an interferon-inducing virus resulted in a higher yield of interferon obtained than with cells not previously treated with interferon. The process was recognised as the first non-antiviral function (Stewart II et al., 1971a). As cells do not seem to require newly induced cell protein synthesis to become primed, the observed effect may be due to a direct alteration of cells. When cells are treated for several hours with relatively large doses of interferons prior to interferon induction, they produce less than cells not previously treated with interferons, Stewart II et al. (1971b). This phenomenon, known as blocking, is unlike priming, in that new cellular protein synthesis is required during interferon treatment for the subsequent development of blocking action. As mentioned above, another action of interferon is enhancement to toxicity of dsRNA. Interferon treated cells are often more sensitive than untreated cells to cytotoxicity of dsRNA

and vaccinia virus, provided the latter is able to synthesise new products, Stewart II et al. (1972a,b).

Historical

Viral interference, the ability of a virus to interfere with the propagation of another, had been known for some time before the activity was named interferon (Lennette and Koprowski, 1946); (Schlesinger, 1959); and (Isaacs and Lindenmann, 1957).

The possible inducers can be classified into two broad categories, (a) viral and (b) non-viral inducers of interferon (Stewart II, 1979).

The first group includes ds or ssRNA or DNA viruses, most species tested induce interferon in animal species only. A number of factors influence the interferon inducing ability of viruses, including metabolic effects of viruses on cells, heat, ultraviolet radiation, and cell culture conditions (Ho and Brenig, 1975). Some known virus inducers include, adenoviruses, myxo-paramyxo viruses (e.g. Sendai Virus).

Non-viral inducers of interferon include, both synthetic and naturally occurring nucleic acids, mitogens and immune recognition (Torrence and De Clercq, 1977). In general, these substances are varied and can induce interferon under a variety of conditions. Most of these non-viral inducers of interferon, especially synthetic dsRNA molecules, e.g. $(I)_n(C)_n$, serve as tools to study mechanism and nature of interferon production, since they are comparatively easier to produce. It has been suggested that synthetic dsRNA might be more useful in medicine than endogenously produced interferons (Finter, 1973).

However, there may be harmful side effects, e.g. $(I)_n \cdot (C)_n$ has a number of known side effects (Lindsay, et al. 1969), such as endotoxin-like effects, pyrogenicity and embryotoxicity (at high concentrations Absher and Stinebring (1969) and Adamson and Fabro (1969)).

Induction of some classes of interferon, such as type II interferon, have not been clearly attributed to any inducers so far. It appears that some cells, constitutively produce interferons (Haase, et al. 1970).

Induction and Production Mechanism

The mechanism of inteferon production has generated much research. Most pertinent information regarding the mechanism of interferon induction arises from the use of $(I)_n \cdot (C)_n$ as the model inducer. Studies have shown that major differences exist between the mechanism of induction by viruses and by dsRNA.

- (1) Kinetics of response of interferon production are different.

Treatment with $(I)_n \cdot (C)_n$ or NDV produces differences in peak responses (Moses and Vilcek, 1975).

- (2) Treatment of chick or human cells with the alkaloid camptothecin inhibits interferon induction by NDV or Sindbis virus, but has no effect on $(I)_n \cdot (C)_n$ induction (Atherton and Burke, 1978).

- (3) Studies on the effect of metabolic inhibitors (e.g. actinomycin D and cycloheximide) show relatively greater resistance of $(I)_n \cdot (C)_n$ induction to these inhibitors (Long and Burke, 1971).

- (4) Pre-irradiation of cells with doses of u.v. produced enhanced interferon production by $(I)_n \cdot (C)_n$ but a decrease in interferon

induced by NDV (Moses and Vilcek, 1975).

It has been recognised by a number of workers that induction of interferons by viruses and dsRNA was accompanied by de novo synthesis of interferon (Tan, et al. 1970), but that the response to polynucleotide induction was of a rather complex nature. It is very likely that both the virus and dsRNA induced interferons are produced from the same gene system, but under different mechanisms of control. This was deduced from the observation that the interferons induced by either agent are antigenically similar (Havell, et al. 1975).

The ability of synthetic double stranded polynucleotides to induce interferon initiated a rational approach to investigations of the requirements of the induction processes, for such RNA species are amenable to chemical modification, allowing detailed studies on structure-function relationships to be undertaken. Variations in modifications have been introduced to the basic structure of the synthetic inducers, allowing for a number of criteria to be defined for a particular species to serve as an interferon inducer. Most inducers are found to possess ds structure of the normal Watson-Crick base paired type, they possess adequate melting temperatures (T_m 's), and are resistant to nuclease under physiological conditions. Generally, complexes with melting temperature (T_m 's) $> 60^\circ$ are the best inducers, T_m 's 40-50 are intermediate inducers, and those below 40 T_m 's are not inducers (De Clercq, 1974). These properties are necessary requirements but not sufficient on their own for being inducers. All inducers possess a minimum 'plug hole' molecular size

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below which inducing ability disappears or is lost. Effective inducing molecular size appears to be 5S and 10S size with the $(C)_n$ strand less sensitive to changes in molecular size than $(I)_n$ strand (Pitha and Hutchinson 1977). However, the possession of one or even all the above properties, does not mean the polymer will be a good inducer. Until recently, it was believed that all inducers possess ribose sugar containing 2'-hydroxy groups in both strands, and various attempts to replace the 2'-hydroxy group in either strand of $(I)_n(C)_n$ or $(A)_n(U)_n$ by a variety of substituents have produced duplexes with no interferon inducing ability (Torrence and De Clercq, 1977). Collectively, these observations appeared to show that either there is a specific receptor determinant for the 2'-hydroxy groups of the dsRNA molecule (Colby, 1971) or that substituents at the 2'-hydroxy group destroy the interferon inducing capability of the polymer due to an alteration of a particular steric configuration which prevents the dsRNA from interacting with interferon-inducer receptor systems. This latter alternative was indeed found to be the case by the interferon inducing ability of an analog of $(I)_n(C)_n$ in which the 2'-hydroxy of the $(I)_n$ strand is replaced by a 2'-azido group. Thus, to induce interferon, the 2'-azido substituents imitated the effect of 2'-hydroxy on the overall conformation of the duplex helix, and, hence, the interferon-inducing property of the complex was not destroyed (De Clercq, et al. 1978).

The only study on requirement for linkage showed that 3'-5' cannot be substituted by 2'-5' for induction of interferon (Pitha and Pitha, 1971), probably because this causes a marked change in shape in the polymer.

A recent study has questioned the validity of high molecular size as a general statement of requirement for interferon induction. Using a series of $(I)_n \cdot (C)_n$ analogues with a differing degree of 2'-O-methylation in each strand, it was found that while high molecular size was important for initial binding to the putative dsRNA receptor, subsequent recognition of helical segments of only 6-12 base pairs could trigger interferon production (Greene, et al. 1978). This observation and the observation that low molecular weight compounds induce interferon synthesis, suggest that interferon induction by different agents may follow different pathways, and that a generalised induction mechanism may be impossible. Many polynucleotide complexes have been synthesised in which structural modifications of the heterocyclic base have led to varying degrees of activity as inducers (Pitha and Hutchinson, 1977). No obvious structural change could be said to result in a predictable shift in inducing ability. However, the circular dichroism (C.D.) study of $(C^7A)_n \cdot U_n$ and $(C^7I)_n \cdot C_n$, (Bobst, et al. 1976), showed that differences in interferon inducing abilities may be due to a major structural change leading to an increase in the positive base tilt in $(C^7A)_n$ and a small decrease of the same parameter in the case of $(C^7I)_n$. While changes in the pyrimidine strand had no effect on the C.D. a dramatic conformational change was observed when changes were made in the purine ring. Thus, it may be the overall spatial and steric organisation of a double stranded RNA that is the determinant of interferon induction. Thus, where base substitution or change leads to loss of interferon induction, it is most likely to be due to an alteration of the duplex conformation sufficient to

account for the non-recognition by the putative dsRNA receptor. Another relevant observation was made by Carter, *et al.*, (1972) who studied a series of duplexes in which degrees of base mismatching have been introduced. Thus, polymers in which deoxyuridine had been introduced into the inosine strand, e.g. $(I_{21}dU)_n.C_n$ and $(I_{39}dU)_n.C_n$, were totally inactive as inducers, whilst the introduction of deoxyuridine residues into the C_n strand produced progressively a decline in interferon inducing ability as the percentage of deoxyuridine was raised. This observation may be explained by the mismatched residues "looping out" of the helices in the non-inducers.

This extrahelical loop may interfere through steric interaction with the proper alignment or approach of the intact unaltered helical segment of the duplex with the receptor. This is supported by observations with mismatched bases (Lomant and Fresco, 1975). It has been hypothesised that the receptor for dsRNA interferon inducers was likely to be a protein (Colby and Chamberlin, 1969), as a nucleic acid receptor would not be capable of recognising the variety of base sequences that are effective inducers. This led Stollar, *et al.* (1978), to suggest that interferon inducers display a particular spatial conformation which is probably the basis for their activity. The antibody systems provide the best evidence so far that the specific receptors for the dsRNA inducers of interferon probably exist at the cellular level. This is concluded from the observation by Stollar's group that antibodies elicited against active inducers show weak cross-reaction with non-inducers dsRNA. The antibody systems are therefore similar to interferon induction, and recognition

depends on the secondary structure and the nature of the sugar-phosphate backbone, and not on a specific base content or sequence of the dsRNA.

Early experiments of Bausek and Merigan (1969) on the dsRNA induction of interferon produced evidence for a temperature dependent step in the induction of interferon, whereby an inducer was susceptible to action of RNase when the cells were kept at 4° but not after the cells had been incubated briefly at 37°. Hence, penetration of the inducer into the cell would occur at 37°, which leads to induction of interferon. At 4°, however, no penetration occurs and no induction. The binding experiment performed by De Clercq in 1974, showed that all classes of polynucleotides, i.e. ss, ds, triplex, RNA, can bind to the cell receptor sites. Triplexes are inactive perhaps because of their many shapes, or they are too tightly bound to cell receptor sites and subsequent treatment with active duplexes does not cause dislodgement from cell surface. The ssRNA's are both too readily degraded at the cell surface, and are reversibly bound. Thus, they cannot induce interferon. On the other hand, dsRNA may effect interferon production by provoking a response that is sensitive to structure of the double helix.

The receptor site of dsRNA has yet to be located, but there are two theories about the dsRNA binding sites. One theory suggests that they are analogous to hormone receptor sites, i.e. selective cell surface site binding occurs, which triggers interferon messenger (Pitha and Hutchinson, 1977) or it may be necessary for penetration to occur leading to productive binding at an intracellular receptor. Indirect evidence for the existence of interferon receptor sites derives from

observations that interferon treatment has been shown to alter the binding of cholera toxin or thyrotropin (TSH) to plasma membranes and to decrease the sensitivity of cells to diphtheria toxin (Friedman, 1977). All these biologically active substances bind to fairly well-characterised, specific receptors. The location, chemical nature and specificity of such putative binding sites have been studied. One study indicated that if such sites exist, they must be on the external surface of the plasma membrane (Vengris et al., 1975).

Indirect evidence for the first theory above, derives from the observation that in one tissue culture study, human fibroblasts were stimulated to produce interferon by dsRNA inducer, $(I)_n \cdot (C)_n$, and inclusion of anti-human interferon antibody in the medium prevented development of an antiviral state, even when the antibody was added after interferon synthesis has already been initiated. This suggests that interferon must be externalised and interacted with the outer surface of the plasma membrane in order to be effective (Vengris, et al. 1975). Friedman (1977), suggested that the external receptors for interferon appear to be gangliosides or ganglioside-like structures with an oligosaccharide moiety at the functional binding site. However, up to date, the first and only direct evidence about the nature of interaction of interferon with cell surface receptors as being the first step in interferon action has been presented by Aguet (1980); studies with 125 I-labelled mouse interferon showed high affinity binding of interferon to a specific cell surface receptor site, and that this binding is an initial step in interferon action. Labelled interferon binds specifically to

interferon-sensitive mouse leukaemia L1210 cells, and mouse L929 cells, whereas binding to interferon resistant L1210 cells and interferon insensitive chick embryo fibroblasts is not specific. That interferon binding shares a characteristic property of other biologically active substances such as hormones and neurotransmitters has been mentioned above, others have shown that interferon action and polypeptide hormones are both transmissible between cells through gap-junctional transfer of secondary messenger molecules (Baron 1966). Blalock and Stanton, (1980) proposed that if the mechanisms of action of interferon and polypeptide hormones are similar, and correct, then interferon should cause a species-specific hormonal response and a hormone should induce tissue-specific antiviral activity. In fact, interferon can cause a species-specific hormonal response (a nor-adrenalin like stimulation of the beat frequency of cellular mouse myocardial cells). Noradrenalin, on the other hand, can induce an interferon-like antiviral state in mouse myocardial cells but not in human amnion cells. These studies led Blalock and Stanton (1980), to suggest that interferon and hormonal action are probably mediated by common pathway(s). Cyclic AMP and/or another small molecule(s) are thought to be generated and transferred after interaction of either group of substances with cell membranes. In the article cited, it was postulated that interferon may be classified as a hormone. It is also possible that these agents share one or more common pathway(s) of action.

On the other hand, a large number of studies have produced a good deal of circumstantial evidence that interferon induction is enhanced greatly by transport of the dsRNA inducer into the cell and hence, by implication, that penetration by the polymer is necessary for activity. Borden, et al. (1978), found that the polyene macrolide amphotericin B, increases interferon production in two cell lines, when incubated with $(I)_n \cdot (C)_n$, presumably by facilitating uptake of polynucleotide. Meager et al. (1978), found a similar increase in yield of interferon when high concentrations of calcium ions were introduced during the induction of various human cell lines with $(I)_n \cdot (C)_n$. Since calcium ions caused aggregation of adenovirus sDNA and increased uptake of DNA into cells, as shown by 100X increases in plaque incidence, the interferon potentials may actually be due to aggregation of dsRNA on cell surface.

The virus induction of interferon is likely to be modulated by dsRNA. All RNA viruses have to produce dsRNA in order to replicate, but in addition, experiments with mutant viruses also suggest that only the virus which produces RNA can produce interferon (Lockhart, et al. 1968), Marcus and Sekellick (1977).

Interferon induction by heat-inactivated NDV was studied in experiments with viruses inactivated by varying the amount of heat, and it was observed that the virus can synthesise interferon if it still has active RNA polymerase (Clavell and Bratt, 1971). This enzyme is of course needed to produce RNA (and hence dsRNA intermediate).

However, such dsRNA do not produce interferon during normal prolific virus replication, since the fully infectious virus also cuts off the protein synthetic apparatus of the host (Sheaff, et al. 1972). Other viral components may well be the inducers in other interferon inducing systems. Studies of interferon production by temperature sensitive mutants of reoviruses, which have dsRNA, suggest a very late step in virus production, maybe the virus particle assembly, trigger s the production of interferon (Lai and Joklik 1973).

A proposed model of virus induced interferon production suggested by De Maeyer-Guignard et al. (1972), indicated that as the virus replicates, it derepresses the cell gene which is concerned with interferon synthesis, and which is normally inactive. The activated gene then forms an mRNA which moves to cytoplasm, and is then translated into an interferon molecule. The evidence for this came from the observation that the interferon found is characterised by the cell (i.e. species specificity), rather than by the virus, and also the process is inhibited by actinomycin D, if added soon after the virus infection. Interferon mRNA has recently been extracted from one species, and then transferred to heterologous species which then produce interferon with the species specificity of the cells from which the messenger was made. This suggests that the hypothesis that mRNA is formed in an induced cell may be correct. Other evidence has accumulated to show that both true induction by derepression mechanism (i.e. agreeing that interferon production is regulated by a repression mechanism), and

production regulation, (due to the cell producing a de-repressor in response to the inducer or inactivation of the cell responsible for destroying interferon or its messages) do occur (Pitha and Hutchinson, 1977).

Stewart II (1979) has recently summarised the various mechanisms of interferon induction and has discussed the three possible mechanisms, namely.

- (i) Double-stranded RNA hypothesis.
- (ii) Repression-depletion hypothesis.
- (iii) Basal-level of interferon hypothesis.

Stewart, however, rules out the double-stranded RNA hypothesis, even for dsRNA induction of interferon. This appears strange since evidences as discussed above, clearly show that dsRNA does induce interferon. The repression-depletion hypothesis which is more favoured by Stewart, suggests that many inducers of interferon disturb protein synthesis and that interferon production serves to protect against this perturbation. If this theory is true, it may explain the induction of interferons by almost all known inducers. But the testing of this hypothesis clearly needs direct experimental evidence.

1.2 MECHANISM OF INTERFERON ACTION

Antiviral Action

The mechanism of the inhibitory action of interferon on viruses and the

subsequent return of cells to normal, are as complicated as the events which led to the production of interferon itself.

Interferon does not interact directly with viruses or affect their adsorption or penetration into the cell. Furthermore, virus replication is blocked at a stage prior to assembly. The maximum interaction of interferon with the cells occurs rapidly, but the antiviral activity usually develops after several hours of incubation (Berman and Vilcek, 1974). Perhaps, in response to the binding of interferon to cell surface, a series of events is triggered that require cellular RNA and protein synthesis. Certainly, the antiviral activity requires de novo cellular protein synthesis and this results in inhibition of virus replication (Taylor, 1964). The length of time needed for attainment of interferon potential has not been established conclusively; Dianzani et al. (1977) reported that when cells are exposed to high concentration of interferon, the cells develop antiviral response much more rapidly than cells exposed to low concentration of interferon. But all these studies show that interferon is implicated in in vivo pathogenesis.

Initiation of antiviral activity will require some metabolic processes, and these processes will be undetectable during the lag period of virus growth. Hence, data on the development of interferon induced antiviral activity must be taken with caution. However, data have accumulated to show that, as mentioned above, cellular RNA and protein synthesis are required to develop

virus resistance, after cells are exposed to interferon (Nichols and Tershak, 1967).

These results have been interpreted as an indication that interferon itself is an inducer molecule and can derepress the host cell genome (including transcription of a cellular mRNA which codes for 'antiviral protein'), and the proteins thus produced inhibit virus multiplication. The putative induced product has been called translation inhibitory protein. However, up to date, no antiviral protein-mRNA has been isolated although one was suggested by Ershov et al. (1977). No antiviral protein has been identified conclusively, though many are being suggested by cell free system studies (these are discussed in Section 1.4.2).

1.3 ANTIVIRAL MECHANISM: SITE OF INTERFERON ACTION

These have been well documented by Friedman (1977) and recently by Stewart II (1979). The data concerning the viral process that is primarily affected by interferon are still somewhat confusing. Several models have been proposed over the past decade for the molecular mechanism of interferon action. The prolific reviews on interferon mechanisms have done little to elucidate the mechanism of action. Interferon has been shown to accumulate whenever the inhibition of virus specific protein synthesis takes place (Friedman, et al. 1972). This and other cell free system studies have indicated that interferon inhibits transcription of viral mRNA in polymerase-containing virus,

possibly by inhibition of viral polymerase function.

Another possible site of interferon action is virus uncoating. Studies with Simian Virus 40 (SV40) seemed to indicate that in interferon treated cells, SV40 uncoating, or a step soon after it, was inhibited (Friedman 1977).

A broad and popular school of thought has it that all the observed effects of interferon are merely amplification of a single mechanism: translation inhibition. This is probably because viral protein and RNA synthesis are so interdependent that it is often difficult to distinguish which of the two is the primary site of action of interferon. This is because progeny RNA molecules are usually responsible for most of the total virus-directed protein and RNA synthesis that is carried on during infection. Therefore, whether or not interferon treatment acts directly to inhibit RNA or protein synthesis early in infection, later, both transcription and translation are profoundly inhibited. The studies on virus translation inhibition have the advantage such that it is possible to prove that interferon action is exerted at the translational levels in two ways:

- (i) Studies of message function input viral RNA in interferon-treated cells.
- (ii) Studies of viral RNA translation in cell free protein synthesising systems (CFS) prepared from interferon-treated cells.

It has, however, not been possible to use either of these two systems to prove that interferon acts exclusively at the translation

level, and the mechanisms of translation-inhibition proposed up to date are very numerous. In addition, cell free systems experiments are difficult to reproduce and the results can be misleading. Caution must be exercised when extrapolating results obtained in such studies, since events in vivo will hardly ever be exactly duplicable in vitro. However, experiments with cell free systems have been of great use in understanding the mode of action of interferon.

Cellular Inhibition of Translation

Viral RNA production is inhibited in interferon-treated cells, as the synthesis of viral RNA depends on specific synthesis of viral RNA polymerase coded by the virus itself. The inhibition could lead from the effect of interferon on translation of viral polymerase from parental RNA, Friedman and Streevalsan (1970), have shown that RNA of temperature sensitive mutant of Semliki Forest Virus, was not able to be translated in interferon-treated cells.

At the ribosomal level, Levy and Carter (1968) speculated that the translation inhibition in Mengo virus-infected interferon-treated cells resulted from the inability of viral RNA to associate with viral ribosomal subunits to form functional polyribosomes. On the other hand, Friedman and Streevalsan (1970) found that association of viral RNA with replication complexes was not inhibited by interferon treatment. Wiebe and Joklik (1975), showed that interferon treated reovirus-infected cells do have viral protein synthesis inhibited, and where the inhibition was high, transcriptional inhibition was

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minimal, thus supporting the notion that translational inhibition is likely to be a major mode of action of interferon.

SV40 is the only DNA virus for which virus-directed protein synthesis in interferon-treated cells has been studied in some detail, and possibly the only virus so far in which the interferon mechanism is thought to operate at three levels: Viral uncoating, viral RNA transcription and translation of SV40 viral cRNA (Friedman, 1977) were inhibited following interferon treatment. The transcription inhibition model of SV40 was however challenged by another experiment which showed that on addition of interferon to a culture of monkey cells, after 24 hours, both early and late SV40 protein synthesis were arrested even though viral mRNA synthesis continued unabated (Yakobson, et al. (1977)). Similar translation inhibitions have been observed in studies with SFV, VSV and Vaccinia Viruses.

One plausible explanation of the interferon induced antiviral mechanism suggests that interferon-treated cells distinguish between cellular RNA and viral RNA by making modified mRNA which differ (structurally or in size) from normal (Levy and Riley, 1973). It was proposed that the need for protein synthesis in interferon-treated cells was to make enzymes needed for the synthesis of this modified mRNA.

While it may be possible for viruses to escape the inhibitory action of interferon at the level of uncoating, and transcription, interferon has been shown to be able to produce substances which can trigger the inhibition of viruses at translational level. Studies of the nature of inhibition produced in cell free systems following

interferon treatment, have led to suggestions about the possible factors which may be responsible for triggering inhibition of protein synthesis after interferon-treatment. However, as will be discussed in the next section, no unifying factor has yet emerged, except for one, the discovery of 2-5A.

1.4.1 INHIBITION OF VIRUS TRANSLATION IN CELL FREE SYSTEM (CFS)

Early methods of detecting viral translation in vitro have been difficult due to the complexity of the eukaryotic cell. The first definite advance was provided by Kerr (1971) who introduced a partially-defined reliable cell free system to study the influence of interferon on virus RNA translation.

Early experiments by Carter and Levy (1967) provided evidence that suggested that interferon induced the production of a new translational inhibitory protein that could combine with ribosomes to inhibit their ability to translate viral (but not cellular) mRNA. However, these studies were directly contradicted by reports from other laboratories, Kerr, et al. (1970) and Content et al. (1975).

1.4.2 DOUBLE STRANDED RNA ACTIVATION OF ANTIVIRAL ACTION

Kerr, et al. (1976) found that after virus infection

in presence of ATP, dsRNA's were prerequisites for the translation-inhibition of interferon-treated cell extracts. But the ATP and dsRNA used, could be removed and the translation-inhibitory activity still remained. Thus, a translation-inhibitory factor(s) has been generated in the reaction medium by a phosphorylation process (Roberts, et al. 1976). It must be mentioned that dsRNA's are formed during either RNA or DNA virus infection. It is therefore possible that activation of translation-inhibition is actually due to dsRNA. Study of the mode of action of the dsRNA mediated translation-inhibition led to the discovery of two interferon-induced, dsRNA-dependent enzymatic activities.

- (i) An oligonucleotide polymerase, which synthesises a series of oligonucleotide containing unusual 2'-5' phosphodiester bonds from ATP (Kerr and Brown, 1978).
- (ii) A protein kinase, which in the presence of ATP and dsRNA, phosphorylates the small subunit of initiation factor eIF-2 (Farrel, et al. 1977). The kinase phosphorylates two proteins at least MWs, 64,000-67,000 and 35,000-38,000, and Histones. MW 35,000-38,000 appears to be the small subunit of the initiation factor eIF-2 needed for protein synthesis.

Several other proteins have recently been found in mouse and human cells upon treatment with homologous interferon. The induction of these proteins by interferon is blocked by actinomycin D (Gupta, et al. 1979).

The interferon mediated kinase has been observed in different

cells too. The activities of the 2-5A synthetase and dsRNA-dependent protein kinase have been purified by adsorption to columns of (I) \cdot (C) $_n$ -Sephadex and eluted with M potassium chloride. Both enzymatic activities can be further resolved by chromatography on DEAE-Cellulose. Interestingly, the kinase-free 2-5A polymerase requires a second component for optimum activity, and the purified kinase activity is no longer dependent on dsRNA (Hovanessian and Kerr, 1979). The potential importance of this enzyme(s) in mediating the multiplicity of effects attributed to interferon has been emphasised (Williams and Kerr, 1980). It is yet to be determined whether or not the kinase is involved in the effects of interferon on transcription or cell growth. However, it is clear that phosphorylation of eIF-2 by the interferon-mediated kinase plays a part in the inhibition of protein synthesis by dsRNA in cell free systems. There is as yet no direct evidence that this inhibition also occurs in the intact cell, but evidence for a pre-activated kinase has been obtained in extracts prepared from interferon-treated mouse L-cells infected with Encephalomyocarditis virus (EMC) and it seems reasonable to assume that the kinase is active in such cells.

The third and most recently discovered class of proteins induced in human and mouse cells after homologous interferon treatment have MWs, 88,000, 67,000, and 56,000, and their synthesis is blocked by actinomycin D, if added together with interferon. The discovery of these proteins is consistent with observations that antiviral effects of interferon require transcription and translation of cellular genetic information. It is not yet established whether all these new

proteins induced by interferon treatment, are related to the two other enzymes (Protein Kinase and 2-5A Synthetase). The activity of the oligonucleotide synthetase (or Polymerase) has been found in other cells, and conditions for efficient synthesis of the inhibitor with dsRNA in solution have been described (Minks, et al. 1979). An enzymatic and chemical analysis of the product synthesised from radioactive ATP (α - ^{32}P , γ - ^{32}P or ^3H) have yielded the structure shown (Fig.1.21)(Kerr, et al. 1978). The biologically synthesised material contains, in addition to the triphosphorylated trimer, variable and smaller amounts of higher oligomers, the significance of which remains to be determined.

The increase in 2-5A synthetase activity upon exposure to interferon has been well studied. Minks, et al. (1979) have shown that in the HeLa cells, after an initial lag of 2-3 hours, the enzymatic activity increases linearly for several hours, and then levels off after 24 hours. The increase over basal level found in control cells varies with different cell lines, but can be as high as 10,000 fold in interferon-treated chick embryo cells (Baglioni, 1979). This increase is dependent on the RNA and protein synthesis, suggesting that mRNA for 2-5A polymerase is synthesised in cells exposed to interferon and that translation of this mRNA is necessary for the increase in this enzymatic activity. This is yet to be documented by isolation and translation of mRNA for 2-5A polymerase. Baglioni, et al. (1979), Ball and White (1978), and Minks, et al. (1979) have shown that a correlation exists between the induction of 2-5A synthetase and the inhibition of viral RNA in EMC-infected HeLa cells, VSV-infected chick embryo cells, and in VSV-infected HeLa cells respectively.

Correlation has been observed in cells exposed to different concentrations of interferon. The 2-5A synthetase in these cells increased with interferon concentration used to treat the cells concomitantly with inhibition of EMCV RNA synthesis, and on treatment of the HeLa cells with interferon, washing off this antiviral agent, the level of 2-5A polymerase decreased with the increase in cell mass in the HeLa cells, while EMCV RNA synthesis is progressively less inhibited. These results suggest the involvement of 2-5A polymerase in the inhibition of viral RNA accumulation and replication.

The product of 2-5A polymerase $(2-5A)_n$ has been shown to activate an endoribonuclease (Zilberstein, et al. 1978). This observation complements an earlier one by Lebleu, et al. (1976) and Sen, et al. (1976). The mixture of extracts of interferon-treated cells with ATP, dsRNA, led to synthesis of endonuclease activity and the phosphorylation of a 67,000 and 35,000 protein. The proteins were then called M1 and eIF-2 initiation factors respectively. In this connection, Galster and Lengyel (1976) have shown that reovirus subviral dsRNA molecules isolated from interferon treated cells were shorter than those from control infected cells, and thus that the endoribonuclease action led to breakdown, and reovirus dsRNA was shown to activate endonuclease in interferon-treated cell saps and ATP. It is generally agreed that the function of 2-5A is to activate an endogenous endoribonuclease which degrades mRNA thereby inhibiting protein synthesis. However, the degradation was not limited to viral mRNA alone, but it was also observed that the endoribonuclease activity was degrading cellular mRNA as well, i.e. it has not been

exclusively shown to be discriminatory. However, recently, Nilsen and Baglioni (1979) have postulated a system by which 2-5A discriminates between viral and cellular mRNA. Using model substrates (poly A-containing mRNA annealed with poly U, etc.), they showed that a localised activation of the endonuclease occurs near the dsRNA region of the model substrates. They proposed that in infected cells, activation of the endonuclease takes place near the replicative intermediates (RI) of the virus being used. The replicative intermediates promote synthesis of 2-5A in extracts of interferon-treated cells and are themselves digested to a 20S 'core' which is resistant to further digestion with RNase. This, they suggested, may be responsible for discrimination between viral and cellular mRNA in interferon-treated cells. Exact relevance of the direct experimental observation of an endonuclease activity is not exactly clear, due to the following.

- (i) As mentioned above, it has not been exactly shown to be discriminatory.
 - (ii) SV40 mRNA is undegraded in interferon-treated cells (Revel, 1977), though it may be argued that endonuclease degradation of viral mRNA takes place after or during association with polyribosomes.
 - (iii) The generation of an endonucleolytic activity in cell saps from interferon-treated cells is not restricted to particular cell species, (Shaila, et al., 1977).
- 2-5A itself is rapidly degraded in the system and the enzyme(s) responsible appears to have similar activity to that of snake venom

phosphodiesterase, yielding AMP and ATP as the digestion product. The degradation is also rapid in absence of added ATP and of an ATP-regenerating system. The responsible phosphodiesterase has recently been isolated and characterised by Schmidt, et al. (1979). The phosphodiesterase called 2'-PDi, has higher activity on 2'-5' than 3'-5' phosphodiester bonds. It splits 2-5A, the oligonucleotide activator of endonuclease (which is thought to degrade mRNA) into 5' AMP and ATP. Interferon-treatment increased the level of 2'-PDi. In addition, the enzyme degrades C-C-A terminals of tRNA, and this reduces amino acid acceptance of tRNA in cell free systems causing a tRNA-reversible inhibition of mRNA translation. The enzyme is a 40,000 MW protein.

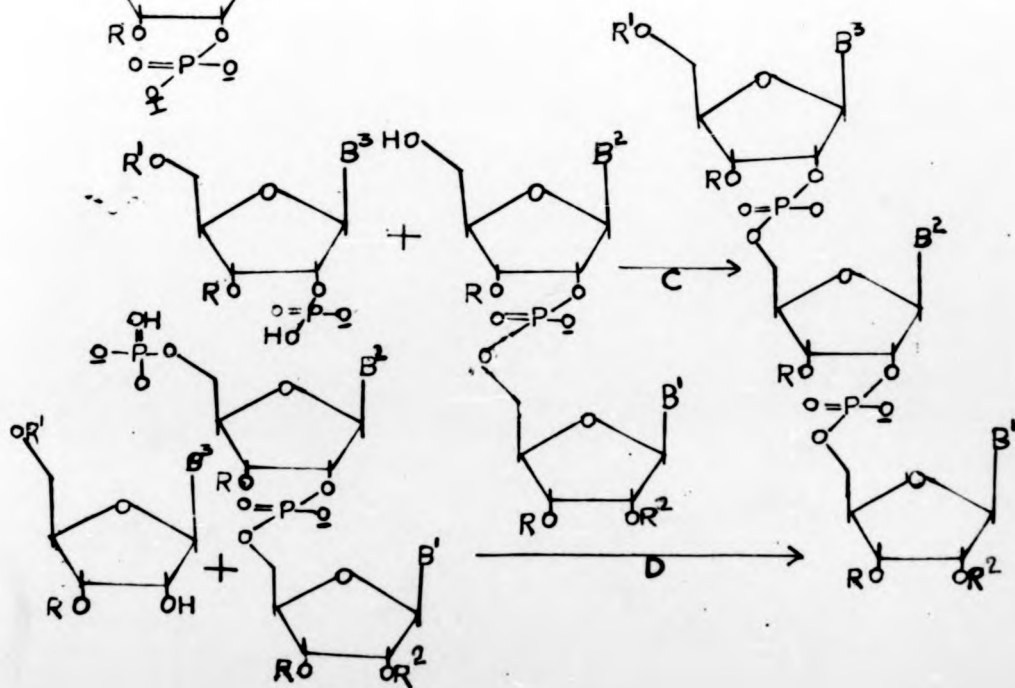
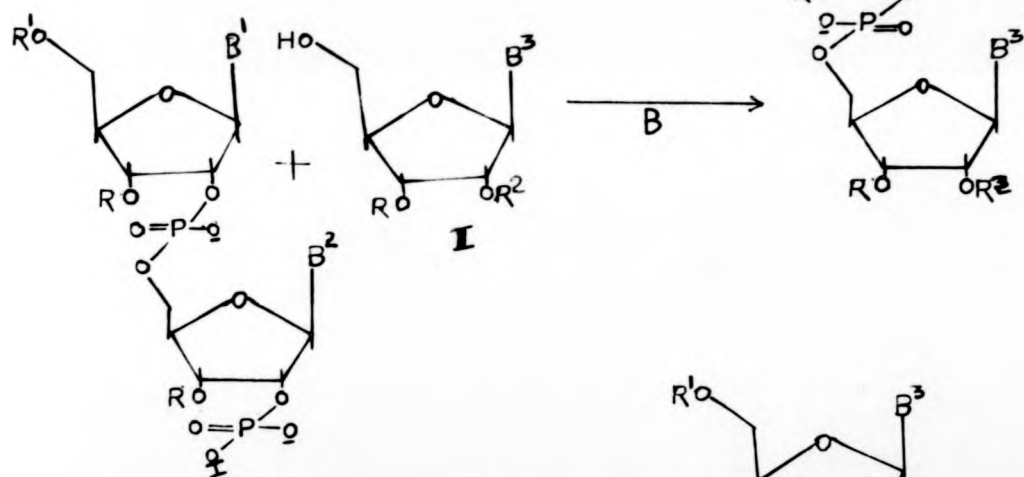
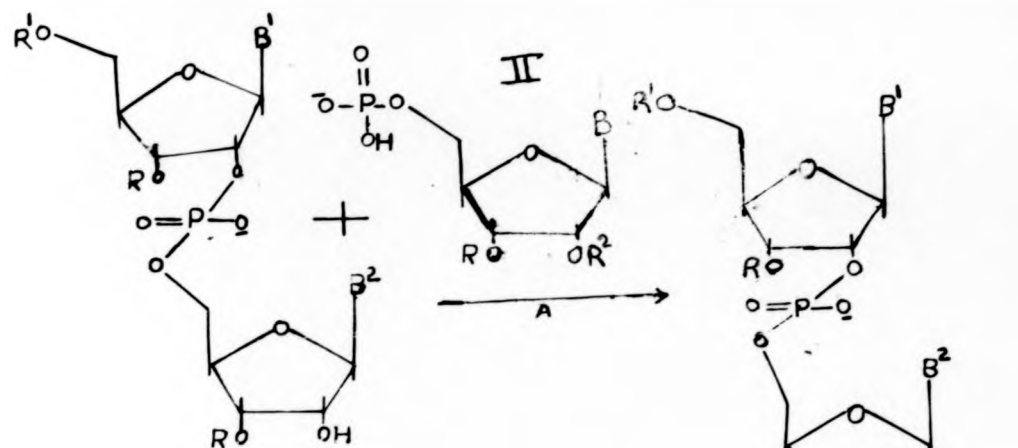
The activity of the 2-5A activable endonuclease is dependent upon the continued presence of 2-5A. Accordingly, it is transient in the absence of a system which regenerates 2-5A (Williams and Kerr, 1980). 2-5A also causes transient activation of a nuclease and inhibition of protein synthesis, when deliberately introduced into permeabilised cells. When introduced immediately after virus infection, it inhibits the growth of virus. It remains to be shown that 2-5A is itself degraded in intact cells. The results obtained in cell free systems and when 2-5A is introduced into intact cells agree with the possible role of 2-5A in inhibition of virus replication using a sensitive biological assay coupled with high pressure liquid chromatographic analysis. The natural occurrence of 2-5A in interferon-treated cells infected with EMC has been detected in (20-200 nmole) amounts sufficient to play a part in the inhibition of virus growth. Enhanced rate of breakdown of RNA in these cells also occurred, this being consistent with

activation of a nuclease by 2-5A present in these cells. Studies have shown that both the endonuclease activated 2-5A and the enzyme responsible for degrading these oligonucleotides, are present in similar amounts in interferon-treated and untreated cells, and that only the enzymic activity which synthesises 2-5A increases dramatically after treatment with interferon (Williams, 1979). Recently, it has been shown by Williams and Kerr (1980), that 2-5A synthetase in cell free systems can add AMP in a 2-5A linkage to a variety of important metabolites as NAD^+ , ADP ribose and Ap_4A , and that this 2'-adenylated NAD^+ is unable to function as a redox co-enzyme. The dephosphorylated core of 2-5A is capable of inhibiting DNA synthesis and cell growth in human lymphoblastoid (Daudi) cells. This is similar to the recently observed effects of interferon on DNA synthesis and cell growth by Creasy, *et al.* (1980). From all these observations made by Williams and Kerr (1980), they suggested that in addition to any part 2-5A may play in the antiviral action of interferon, it may also be involved in the control of normal cell growth and development, particularly where mRNA degradation may be a requirement in preparing the cell for a specialised function, thus suggesting a wider role for the 2-5A system. This claim needs further investigation, and whether the inhibition of DNA synthesis and cell growth by 2-5A are secondary to an initial effect on protein synthesis, or represents a quite independent response to the 2-5A system remains to be determined. It will be interesting to determine firmly the regulatory role of the 2-5A system. But, it is clear up to date that either 2-5A itself or the synthetase, may have a wider signifi-

FIGURE 1.1 Four possible elongation routes to oligonucleotide
synthesis by phosphodiester approach.

B = Nucleic Acid Base.

R = Protecting Group.



cance in regulating normal cell growth and development.

1.5 CHEMICAL SYNTHESIS OF OLIGONUCLEOTIDES

1.5.1 INTRODUCTION

Substantial progress has been made in the area of stepwise synthesis of oligonucleotides, and this has been well reviewed by

- (i) Amarnath and Broom (1977)
- (ii) Reese (1978)
- (iii) Ikehara, et al. (1979)

The synthesis of oligoribonucleotide is more complicated than the synthesis of oligodeoxyribonucleotide, as the presence of two secondary hydroxy groups in the ribose moiety of the ribonucleosides profoundly influence their chemistry and complicate the problem of synthesis of both 3'-5' and 2'-5' linked oligonucleotides.

However, within the past decade, there have been major developments which have improved the chemical synthesis of oligoribonucleotides. These developments include the diester approach of Khorana, which has contributed to synthesis of gene sequence (Khorana, et al. 1976, et seq.), and the triester approach developed by Letsinger (Letsinger and Mehadevan, 1965). These

developments have allowed modest progress in the synthesis of ribooligonucleotides, thus they have allowed the deciphering of the genetic code, and allowed extensive tests of oligonucleotides on stimulation of the binding of the different aminoacyl tRNAs to

ribosomes. However, the efficient procedures for the chemical synthesis of ribooligonucleotide of defined sequence are still being developed. All advances so far made involve the synthesis of 3'-5' linked ribooligonucleotide, since until recently, this is the only linkage involving nucleotides in natural nucleic acids.

2-5A has been isolated (see previous section) and it was our intention to synthesise the molecule to confirm its structure and to obtain sufficient material for detailed biological studies.

There have been various attempts to synthesise this unusual oligonucleotide chemically. However, the yields are often not reported, and when reported, are often low. Some of these approaches are discussed in Section 1.6.

In this thesis, synthesis of pentaphosphorylated trimer (2-5A), via the modified Khorana's diester approach, previously used in synthesis of oligonucleotide with 3'-5' linkage is described. The core trimer was phosphorylated by purified wheat shoot enzyme (see Chapter 2), characterised chemically and enzymatically, and some of its biological properties in cell cultures studied.

Whether the synthesis of oligonucleotide is to proceed via the phosphodiester or phosphotriester approach (see below), there are six major problems.

- (1) The selection of suitable protecting groups for the hydroxy, amino and phosphate groups.
- (2) The synthesis of nucleosides protected on the 3'-hydroxy and/or on the 3'- and 5'-hydroxys (for synthesis of 2-5A).
- (3) The condensation of the protected nucleosides or

nucleotides and chain extension techniques.

- (4) Efficient developments of the phosphorylation reaction.
- (5) Efficient deblocking methods, to give good yields of the final product.
- (6) The need to use phosphorylated intermediates with unprotected internucleotide linkage, i.e. the phosphodiester approach, has been very successful only for the deoxyribose series, as there is no chance of participation of the 2'-hydroxy in the reaction. This approach is discussed in the next section.

Usually, several steps are needed to satisfy requirement (2) and the yields are generally low (Reese, 1978). Moreover, the condensation reactions are usually slow. However, recently, van Boom, *et al.* (1977), did report a yield of 40% in condensation of dinucleoside monophosphate. The techniques for phosphorylating oligonucleotides are generally inefficient. This problem is described in Chapter 2. Finally, the alternative to (6) above involves prior protection of the internucleotide linkage, hence is called phosphotriester approach. Scheme 1.20 shows an example of such an approach. The problems listed above (1-6) are individually investigated in some depth in this project; while the work was in progress, other syntheses appeared.

The major difference between the phosphodiester and phosphotriester approaches lies in the presence of protected phosphodiester bond (triesters), which affects the methods of condensation and purification of the condensed products. The inherent disadvantages of the diester technique have been succinctly listed by Reese (1978) as follows:

- (a) Phosphodiester functions are nucleophilic and are open to attack in subsequent phosphorylating steps. The

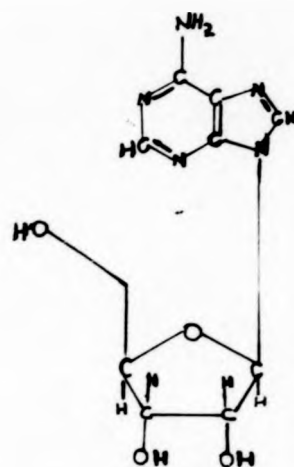
side-reaction would upset the stoichiometry, possibly resulting in cleavage of the internucleotide linkage and generally lead to lower yields. Hence phosphorylating species formed could attack the internucleotide phosphodiester linkage as well as the free secondary alcohol function.

- (b) Salts of phosphodiesters are normally soluble in water, and the more polar organic solvents. They are therefore not usually easily purified by the standard technique of organic chemistry, e.g. adsorption chromatography on silica gel; fractionation techniques such as ion-exchange chromatography must be used.

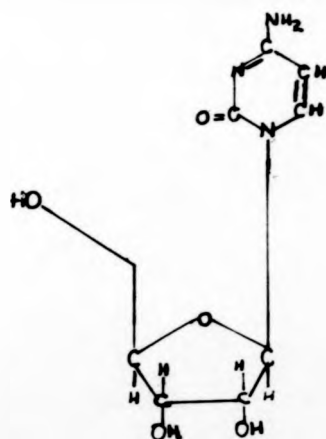
Hence, care must be taken in purification of partially protected phosphodiester intermediates as the protecting groups used are necessarily sensitive to either acid or to base catalysed hydrolysis. The purification of phosphodiester intermediates is complicated in the ribose series as it is necessary that no unblocking of the 3'-OH function occurs during subsequent concentration of fractions.

The improvement of the above problems is based on the protection of internucleotide linkages, hence the phosphotriester approach. The greatest drawback in the later approach is in the choice of suitable protecting group for the internucleotide linkage. This is discussed under condensation techniques.

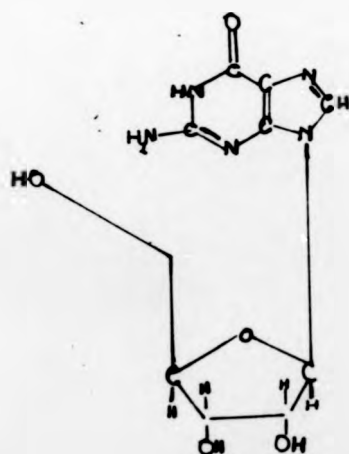
- FIGURE 1.2 (a) Adenosine (9- β -D-ribofuranosyladenine)
(b) Cytidine (1- β -D-ribofuranosylcytosine)
(c) Guanosine (9- β -D-ribofuranosyl(guanine))



ADENOSINE



CYTIDINE



GUANOSINE

Fig. 1.2

1.5.2 PROTECTING AGENTS FOR FUNCTIONAL GROUPS

1.5.2.1 PRIMARY AMINO FUNCTION

Protection of the amino functions of adenine, guanine, cytosine (see Fig. 1.2) is essential for high yields. However, two or more additional steps are needed (blocking and unblocking). The protection however leads to improved solubility when the heterocyclic extranuclear amino groups are masked. However, there have been some reports that the lack of protection of the primary amino functions does not lead to side reactions, even when powerful phosphorylating agents like TPS-tetrazole, are used. Reese, *et al.* (1968), reported that adenylyl (3'-5')uridine is obtainable without prior acylation of the amino functions. While Narang, *et al.* (1972), observed N-phosphorylation of free amino groups, Smrt (1978), reported the apparent superfluity of amino protecting groups in adenosine and guanosine (Fig. 1.2) and it was found that the yield and purity of the dinucleotide were not affected by the presence or absence of blocking agents in amino functions. Essentially, the choice of the N-protecting group depends on the experience, need and properties of nucleoside being handled by a particular research worker. Since the literature has not clearly defined measures for choices of an individual worker, one has to presume that the choice depends on the above factors. However, it is generally agreed that the protection of amino functions will not have any undesirable side effects, and that lack of such protection may result in a series of side effects as detailed above.

There are not many amino protecting groups developed, but the general approach is to N-substitute the bases before forming internucleotide linkages. The N-substituents can be removed after the synthesis of the internucleotide bond, hence the N-protecting group must be stable to all chemical manipulations during an oligonucleotide synthesis; its removal must not affect the phosphodiester bond. For the synthesis reported here, the benzoyl group, an acyl protecting group was chosen.

Acyl Groups

N-acylation(acetyl, benzoyl, anisoyl) is most frequently used as method of protection. The general strategy is to fully acylate the mononucleotide or nucleoside and then selectively liberate the hydroxy groups by taking advantage of the facile base hydrolysis of esters compared to amides. Also, aromatic amides are known to be more stable than aliphatic amides at high pH, due to ionisation of the N-H group which may be an additional reason for the preferential hydrolysis of the O-acyl over the N-acyl groups. Excellent yields of the protected products, e.g. N-benzoyl deoxyadenosine have been obtained. A major disadvantage of N-acyl derivatives is that the glycosidic bond is made more susceptible to hydrolytic cleavage. The derivatives also have limited solubility in pyridine, and the acetylated derivatives are unstable in acidic media, these factors restrict their use. However, a number of useful N-acylated derivatives have been prepared. In general, different acyl groups are used for different bases.

- (i) Anisoyl group is often used for protecting the amino function of adenine and cytosine (Ralph and Khorana, 1961).
- (ii) Benzoyl group is often used to protect the amino

functions of adenine (e.g. Markham, et al. 1979).

- (iii) Isobutyryl or 2-methoxybutyryl group has been used for protecting the amino functions of guanine (Büchi and Khorana, 1972).

The acyl groups are removed by treatment with concentrated ammonia solution or with 1:1 mixture of methanol and butylamine (Weber and Khorana, 1972). Solubility of protected derivatives is often a major problem in oligonucleotide synthesis, and N-acyl groups have different solubilities. Thus p-alkylbenzoyl which is more lipophilic and hence more stable, than benzoyl groups, may be preferred.

1.5.2.2 HYDROXY FUNCTIONS OF CARBOHYDRATE

Protection of the carbohydrate moiety of nucleoside is more important in ribose than in the deoxy series. Firstly, the stereochemical environment of the primary alcohol on one hand, and two different secondary alcohol functions of ribonucleosides, are different; on the other hand, it will be easier to distinguish between the primary and secondary alcohols in deoxyribonucleosides. The migratory tendencies of acyl groups from one of the secondary hydroxy functions to the other in ribonucleosides complicates the synthesis of oligonucleotides. Factors to be considered in choosing protecting groups for the hydroxy functions of a carbohydrate moiety are as follows:

- (a) The group must be specifically and easily attached to the hydroxy group to be protected.

FIGURE 1.3

(A)

(a) DCC

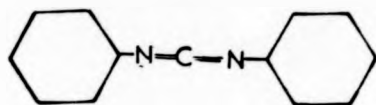
(b) TPS-Cl

(R = isopropyl, x = chloride)

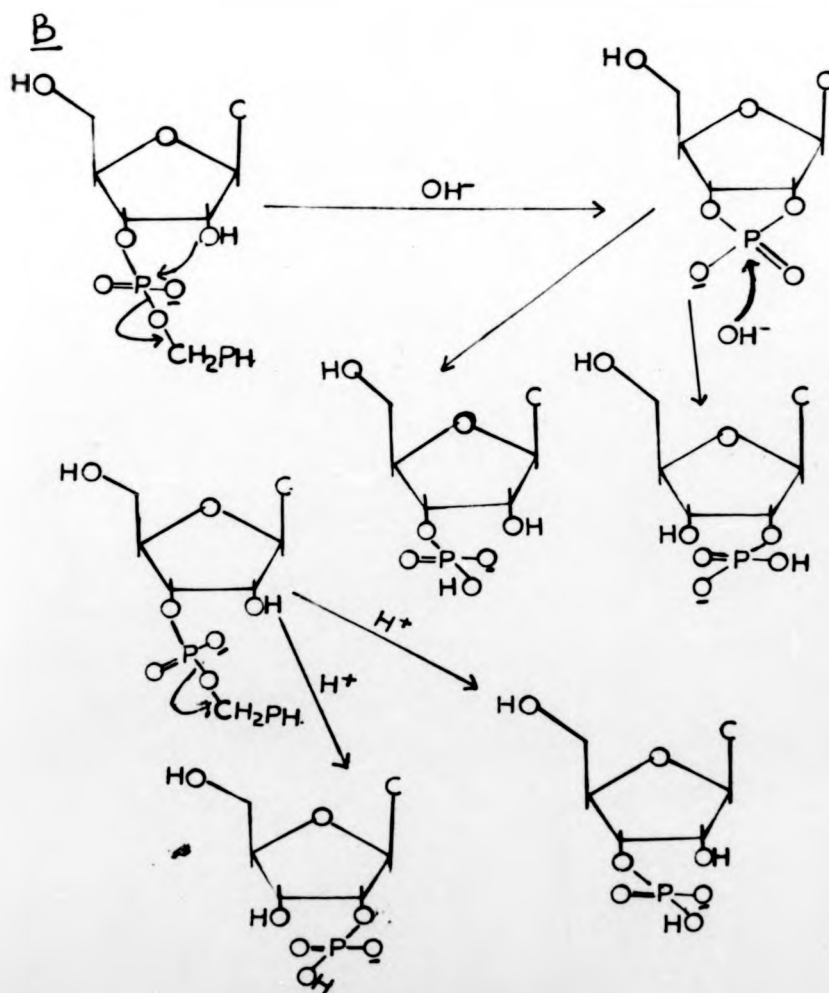
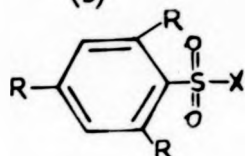
(B) Acidic and alkaline hydrolysis of cytidine
3'-benzyl phosphate in hydrochloric acid and
alkali (Brown and Todd, 1953).

A

(a)



(b)



idine
acid and

- (b) Stability of the group to the conditions to be used for internucleotide bond formations.
- (c) Deblocking or removal of the protecting functions must not lead to 2'↔3'-isomerisation or breakage of phosphate linkage. Similarly, the effects of blocking and deblocking processes on the overall yields and ease of crystallisation of products must also be considered.

PROTECTION OF THE 2'(-3')-HYDROXY GROUP

One of the crucial decisions that has to be made in the synthesis of oligonucleotide is the protection of the 3'(-2')-hydroxy with a protecting group which will remain intact until the very last step of the synthesis and whose removal will not lead to isomerisation and the release of vicinal phosphodiester groups. Brown and Todd (1953) have shown that when benzyl ester of cytidine 3'-phosphate was heated in 0.1 M hydrochloric acid solution at 80° for 2 hours, 50% hydrolysis to mixture of cytidine 2'- and 3'-phosphate occurred and the unhydrolysed product contained the benzyl ester of cytidine 3'-phosphate and some of the isomeric 2'-benzylester (Fig. 1.38). Thus, acidic conditions lead to hydrolysis and phosphoryl migration. Alkaline hydrolysis of the benzyl ester of cytidine 3'-phosphate, gives the cytidine 2'-phosphate and cytidine 3'-phosphate (Fig. 1.38).

(a) Acid Labile Groups

The difficulty of separating 2'-5' from 3'-5' linked oligonucleotides by ion exchange chromatography makes it pertinent to avoid phosphoryl-migration during the removal of 2'-hydroxy protecting group. It seems wise therefore that if the acid labile protecting group is to be used, it must be removable under mild conditions. Some work has been reported on studies of the lowest level of acidic conditions that will not lead to phosphoryl migration. Studies on the use of the tetrahydropyranylacetal system on isolated 2'-hydroxy functions led to the conclusion that the system is not satisfactory for the protection of secondary alcohol functions (Smrt and Sorm, 1962).

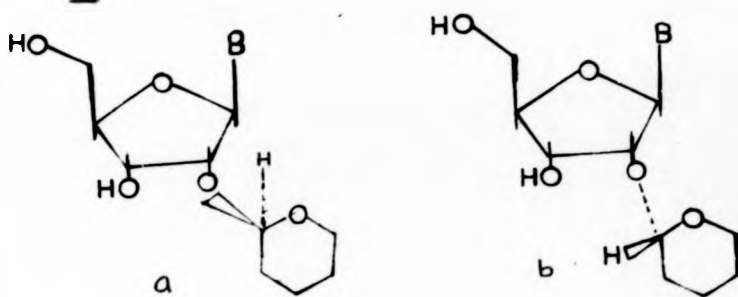
The reaction of 2,3-dihydro-4H-pyran with an asymmetric nucleoside gives rise to a pair of diastereoisomers (Fig. 1.4A) in a ratio which is determined in an unpredictable manner by asymmetric induction. In addition to the obvious reduction in yield inherent in the selection of only one isomer, a tedious separation procedure is required in order to obtain a pure, crystalline, blocked nucleoside (Amarnath and Brown, 1977). In addition, the (2' and 5') protected bis(methoxy-tetrahydropyranyl) derivatives have been prepared. The enol ether reagent (2-methoxytetrahydropyranyl function) has been successfully used for the synthesis of oligonucleotides by van Boom, et al. (1971).

(b) Trityl Groups

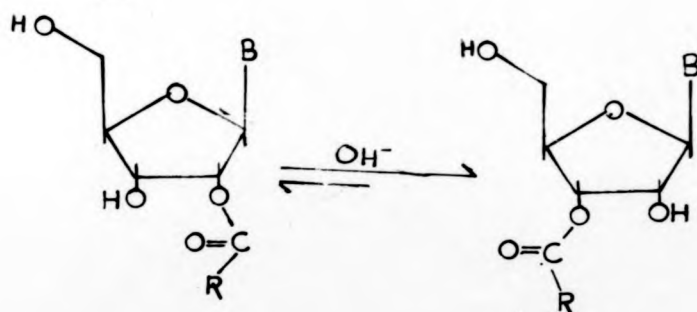
The first use of trityl groups as nucleic acid protecting groups, was by Khorana. It has been found useful for blocking 2'-hydroxyl function, e.g. 2',5'-di-O-trityluridine was synthesised

- FIGURE 1.4
- (A) Synthetic disadvantage of 2,3-dihydro-4H-pyran reacting with asymmetric nucleosides. Pair of diastereomers (a) and (b) in an unpredictable manner by asymmetric induction.
 - (B) Base-catalysed acyl migration in 2'-~~O~~-acyl derivatives of ribonucleotides.
 - (C) Pixyl chloride (9-chloro-9-phenylxanthene).

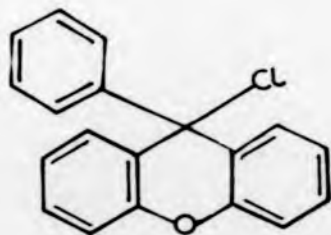
(A)



(B)



(C)



by Cook and Moffatt (1967). The bulkiness of the substituted trityl groups makes them less useful at the 2'-hydroxyl position, and their optimum uses involve the protection of the primary alcohol group (Hall and Thedford, 1963). This group has been extensively used by various workers and also in this project, to protect the 5'-hydroxy (see Section 1.8.3).

(c) Base Labile Groups

In his Nobel Prize winning contributions to nucleic acid chemistry, Khorana and co-workers (Rammner, Lapidot and Khorana, 1963), used the base-labile acetyl and benzoyl groups for protection of the 2'-hydroxyl group. These are the most investigated acyl-protecting groups. Fromageot et al. (1968), showed that 2'-acyl derivatives of cytidine and guanosine can be prepared. However, base catalysed acyl migration to give a mixture of 2' and 3'-isomers in some nucleosides, limits their uses for 2',3'-OH protection (Reese and Trentham, 1965). The equilibrium seems to favour the 3'-isomer, migration being slower in anhydrous pyridine, and very fast in aqueous solution at pH 7.0. Other less mobile acyl groups have been used in protecting both the 2' and 3'-hydroxyl function, e.g. 2',3'-O-alkoxyalkylideneadenosine derivatives. Upon mild acid treatment, these compounds will yield mixtures of 2' and 3'-O-acylribonucleosides, but as mentioned before the 3'-isomer predominates, and the separations are somewhat laborious. The benzoyl, chloroacetyl, methoxy acetyl, and formyl groups have been developed and used in synthesis of short chain oligoribonucleotides.

The mild base lability of acyl groups has been combined with enzymatic techniques to develop an important component of oligoribo-

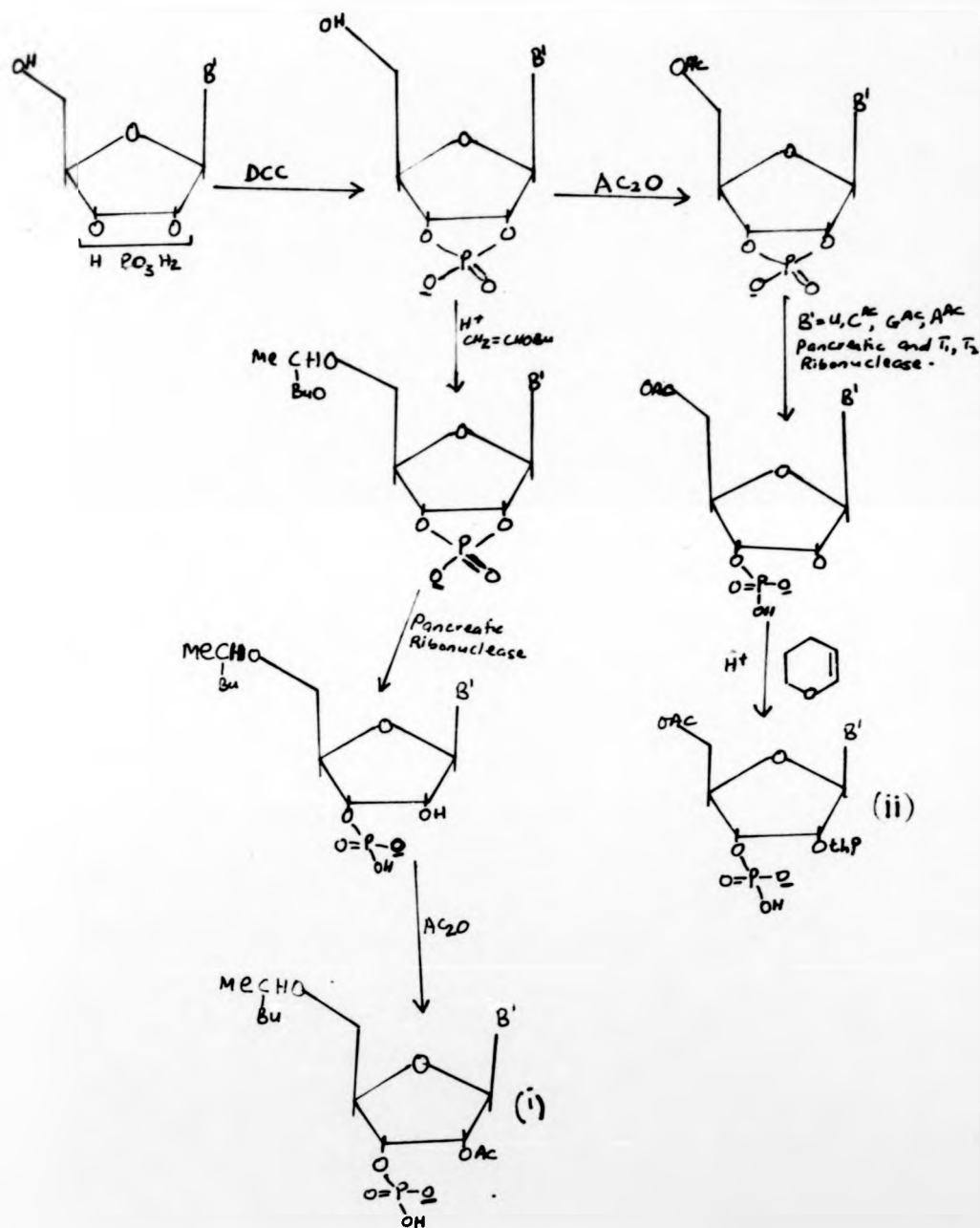
FIGURE 1.5 Route to key components in oligoribonucleotide synthesis:
Synthesis of 3'-nucleotide bearing substituents at 5'-
and 2'-sites which are selectively removable under
different conditions.
(i) and (ii) are synthesised by combined chemical and
enzymatic approaches.

otide synthesis:

tuents at 5'-

ble under

chemical and



$\text{B}' = \text{N-acetylated base.}$

nucleotide synthesis (Fig. 1.5) (Ohtsuka, et al., 1971). Other 2'-protecting groups removable under conditions different from above include the following:

- (i) 2'-O-benzyl groups, were used as building blocks in synthesis of UpU (Griffin and Reese, 1968). The protecting group is removable by catalytic hydrogenation. Whereas no hydrogenation of 5,6-double bond of uracil was observed by Griffin and Reese (1968). Peitz and Pfeleiderer (1975) observed 10% hydrogenation of uracil residues.
- (ii) Photochemically removable O-nitrobenzyl group has been employed by Ohtsuka, et al. (1974)
- (iii) Ogilvie and Theriault (1974) have used di-*t*-butyldimethylsilyluridine in synthesis of UpU, this protecting group can be removed with tetrabutylammonium fluoride in tetrahydrofuran solution. The protecting group has been used in synthesis of 2-5A (Ogilvie and Theriault, 1979) (also see section 1.6).

STRATEGIES FOR ELONGATION OF THE OLIGONUCLEOTIDE CHAIN

The stepwise elongation of an oligonucleotide chain depends on the actual building blocks used for the general synthesis, which in turn depends on the protecting groups which are to be used. From experience, we found that it is easier if the latter is chosen to satisfy the precise building blocks required. Possible elongation steps, using phosphodiester approach, are shown in Fig. 1.1. Groups labelled R¹ remain intact to be removed at the final stage,

and groups labelled R^2 must be selectively removed before each elongation.

Chain extension in which acid labile 2'-hydroxy protecting groups will be used, may be accomplished using terminal units I and II shown in Fig. 1.1, in which the 3'- or 2'-hydroxy groups are protected by acid labile groups. In the phosphotriester method, chain extension along the 3'- end or the terminal 5'-hydroxy function, will need 2',3'- and 2',5'-protected non-terminal or chain extending building blocks. These are the general strategies of the triester approach. In the diester method, since the 5'- or 2'-hydroxy groups (in the synthesis of 2-5A) normally bears the phosphate moiety, it is only one of the secondary hydroxy functions (3'-) and/or 5'-hydroxy that need protection. Terminal protected chain building blocks could then be either of the following.

- (i) Acid-labile group for 2'/3'-hydroxy, if the starting material is 5'-adenylic acid. OR
- (ii) Using similar reagents on the 3'/2'-hydroxy, e.g. a cis-diol group, which is stable to subsequent treatment (see section on acid hydrolysis of isopropylidene adenosine) and removable at the final stage.

Synthesis of non-terminal units for phosphodiester method involves:

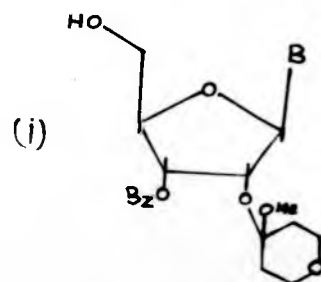
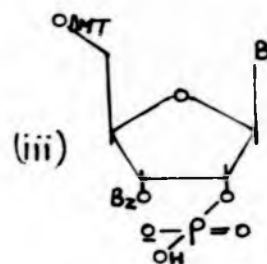
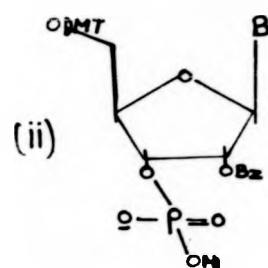
- (a) acid labile group for 5'-hydroxy and base labile 2'-hydroxy protecting groups, if the starting material is 3'-adenylic acid,

FIGURE 1.6 Non-terminal ribonucleoside building blocks.

2',3'-protected non-terminal building blocks:

2'-acetal-3'-ester

- (i) 2'-acetal-3'-ester of ribonucleoside
- (ii) 2'- ester-5'-ether of ribonucleotide
- (iii) 3'- ester-5'-ether of ribonucleotide



Bz = PhCO—

- (b) acid-labile group for 3'-hydroxy and base labile group for 2'-hydroxy if the starting material is 5'-adenylic acid (Fig. 1.6).

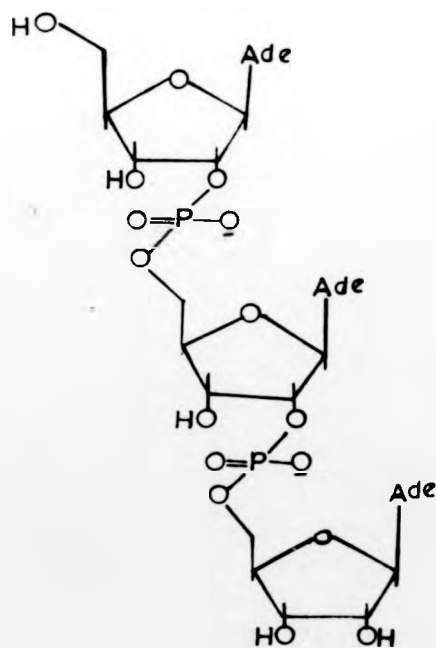
PROTECTING GROUPS FOR 5'-HYDROXY FUNCTIONS

In the general strategy discussed above, some of these protecting groups have been mentioned. Only the class relevant to this thesis will be discussed. The reader is advised to consult page 28 for appropriate reviews on those groups not discussed here.

Acid Labile Groups

Trityl, mono-, di-methoxytrityl groups: These groups have been extensively used in oligonucleotide synthesis by the diester method (Ogilvie, et al. 1977). These workers have also suggested their usefulness in the triester approach. Trityl chloride derivatives are the most commonly used blocking agents for the 5'-hydroxyl function. Methoxy substituents at the para-position(s) of one or more benzene rings render these reagents more reactive than unsubstituted trityl chloride, and make the substituted trityl chloride slightly less selective towards primary alcohols (Armanath and Broom, 1977). While this represents a setback to the use of the methoxy substituted trityl chloride derivatives, this is more than compensated for by the relative ease of removal of the substituted derivatives. Acidic conditions necessary for hydrolysis of the unsubstituted trityl group (see section on acid hydrolysis experiments) are severe enough to cause an isomerisation of the phosphodiester bond in the ribose series and cleavage of the glycosidic linkage in the 2'-deoxy series. Thus, the unsubstituted derivatives may

FIGURE 1.7. Synthesis of 2-5A via phosphodiester approach.



be useful in the triester approach, since the phosphate should be more stable to acidic hydrolysis, but as mentioned earlier, deblocking of the triester to give final product, often produces a low yield.

The labilities of mono, di-, and tri-p-methoxy substituted trityl derivatives increased by a factor of 10 respectively in 80% acetic acid or when treated with naphthalene radical ion in hexamethylphosphoric triamide (Greene and Letsinger, 1975), and also this thesis, Section 1.8.9); hence the monomethoxy substituted trityl derivatives are most stable and they are often used for 5'-OH protection. Fig. 1.7 shows the use of 5'-dimethoxy trityl in synthesis of a trinucleoside diphosphate.

Advantage of the use of trityl derivatives lies in their ease of detection by means of ceric sulphate spray (Neilson and Werstiuk, 1971) and in addition the presence of the bulky trityl group makes the nucleotide chains lipophilic and hence more soluble in organic solvents. Recently, other groups have been introduced which have properties similar to the trityl chloride and have acid lability similar to that of the substituted trityl group, e.g. the pixyl derivative (9-phenylxanthen-9-yl) have been used as protecting group (Chattopadhyaya and Reese, 1978) (Fig. 1.4c). This was introduced due to difficulties in crystallising the substituted trityl nucleoside derivatives. Similar problems relating to crystallisation of the nucleoside trityl derivatives have been reported by Schaller et al. (1963). Similarly see section 1.8.3 of this thesis. However, the pixyl derivatives were found to be too labile unless very dry solvents were used. This poses a setback to the use of this possible 5'-protecting group.

FIGURE 1.8 Synthesis and use of MPDA.

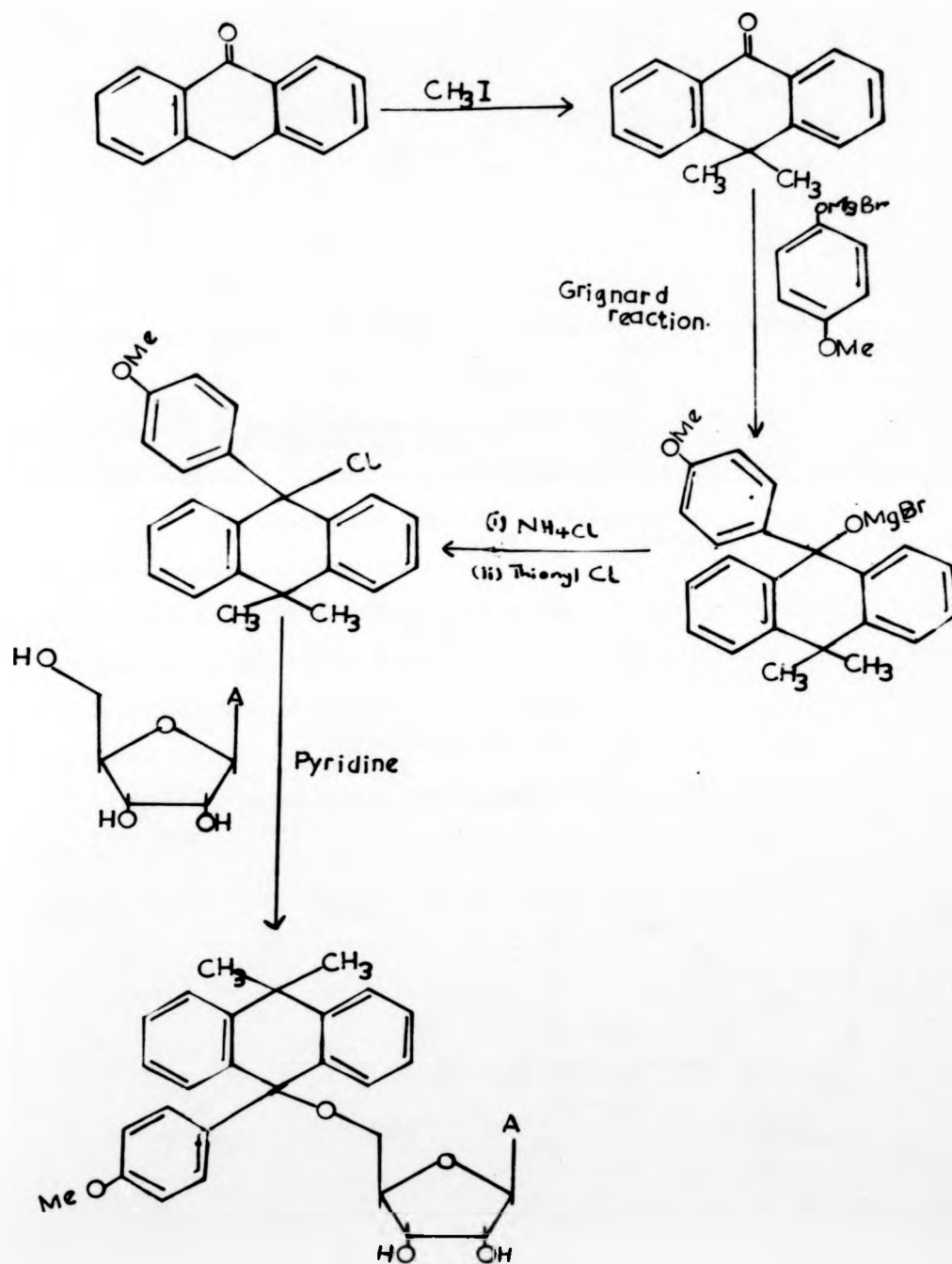
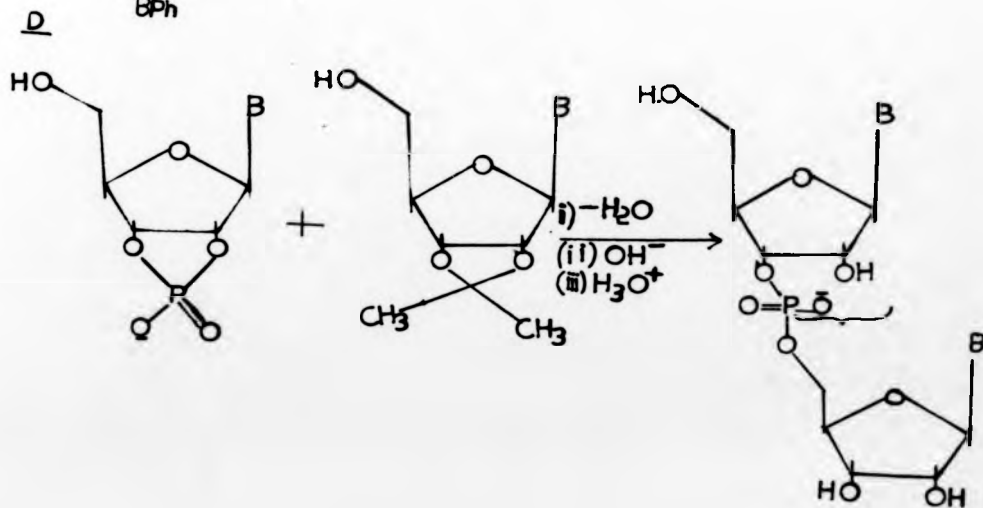
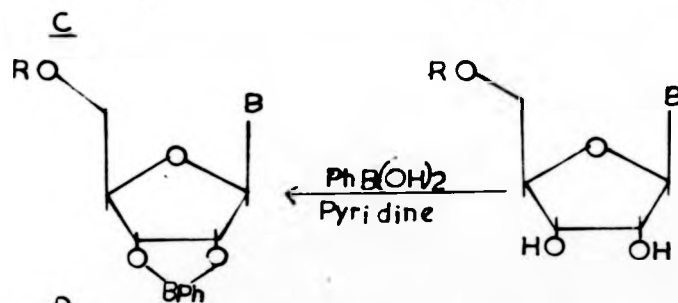
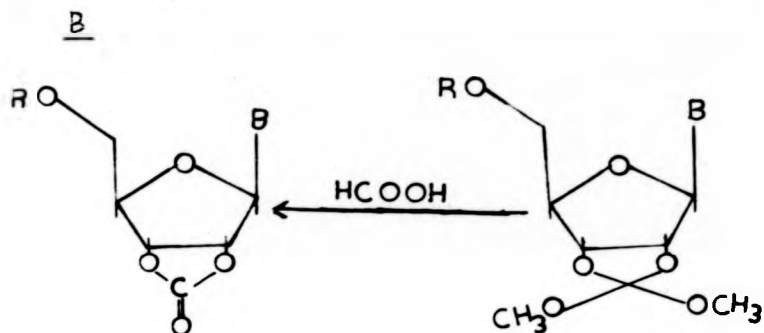
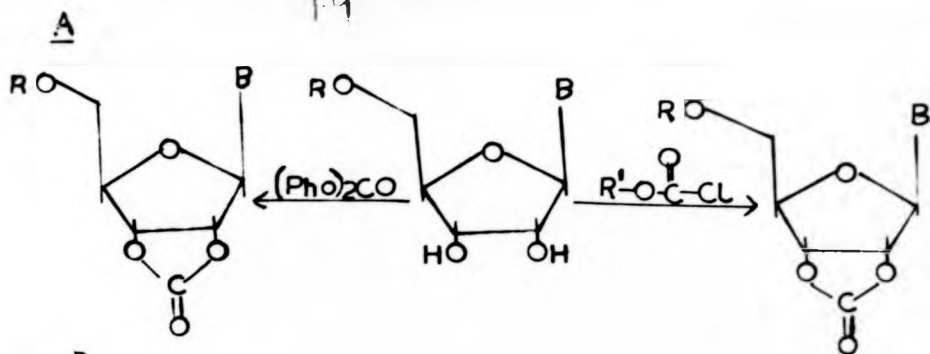


FIGURE 1.9

Structure and uses of 2',3'-cis diol protecting groups.

- (A) 2',3'-O-cyclic ester.
- (B) Dimethoxymethylidene, used as a source of cyclic carbonate.
- (C) 2',3'-O-phenyl boronates.
- (D) Synthesis of non-specific phosphodiester linkage using 2',3'-cyclic phosphate with a 2',3'-isopropylidene acetal of a nucleoside in presence of diphenylchlorophosphate and tributylamine. A mixture of 3'-5' and 2'-5' linked dinucleotide monophosphates are produced.



With the above potentials and limitations of the trityl derivatives in mind, we fashioned a protecting group in the mode of pixyl chloride namely MPDA (Fig. 1.10). This novel protecting group, and the advantages and limitations of the different 5'-protecting groups will be discussed as we observed them during this project.

TERMINAL 2',3'-PROTECTED UNIT

This is very useful for the synthesis of oligonucleotides from the 3'- or 2'-end, mono- or oligo-nucleotide blocked at its 5'-end (in phosphotriester) or 5'-phosphate end in the phosphodiester approach. Also, when the synthesis in the opposite directions is terminal, both 2'- and 3'-hydroxyl functions at the 3'-terminal must be protected.

The selectivity for the cis-glycol system may be readily achieved by taking advantage of the stability of 5-5 fused ring systems. A variety of reagents which react with the cis-diol group to form five-membered dioxolane-type rings have been used.

2',3'-O-alkylidene Derivatives

One of the earliest developed 2',3'-cis-diol protecting groups, is the isopropylidene group. Today it is still widely used, and we have found it useful in the synthesis of 2-5A and its acid stability with others studied are discussed in section 1.8.9. Other alkoxyalkylidene derivatives or cyclic orthoformate esters (e.g. methoxymethylene acetate) have been prepared by the reaction between the ribonucleoside and methoxymethylidene or

trimethyl orthoformate (RC(OMe)_3) or thioester. Diastereoisomers are formed, but these acetals can easily be hydrolysed under relatively milder conditions and are preferred to the isopropylidene group. However, treatment of these acetals with aqueous acid, results in 2'-(3'-)acylates, and only alkaline treatment ensures complete cleavage. This makes them less useful when alkali labile groups exist on the other part of the protected oligonucleotide. Other varieties of alkylidene derivatives have been prepared via other ketones (instead of acetone used in isopropylidene synthesis) and aldehydes. The use of benzaldehyde leads to a mixture of diastereoisomers, benzylidene acetals. The isopropylidene and benzylidene acetals are too acid stable ($t_{1/2} = 19.7$ hours, in 80% acetic acid for isopropylidene adenosine, unpublished observation), and may result in migration of phosphate $2' \rightleftharpoons 3'$, hence more acid labile groups have been introduced. These include the following.

- (i) 2,4-Dimethoxy benzylidene acetals.
- (ii) 2',3'-O-Cyclic esters.
- (iii) 2',3'-Cyclic phosphates.
- (iv) 2,3'-O-Phenyl boronate.

Structures and some of the uses of these esters are shown in Fig. 1.10b.

1.5.23 PHOSPHATE PROTECTING GROUP

The blocking of a terminal phosphate residue from further reaction either intramolecularly to form cyclic phosphate or intermolecularly to form pyrophosphate is absolutely essential in the triester approach. Various internucleotide groups have been synthesised and used, these include the following.

- (i) 2,2,2-Trichloroethyl group. The trichloroethyl phosphate is generally coupled to a free hydroxyl group by using DCC or TPS-Cl. The protecting group is usually cleaved by zinc dust and 80% acetic acid.
- (ii) 2-Cyanoethyl group. This is still often used to protect phosphate moiety in triester method.
- +(iii) Phenyl and substituted phenyl moieties (e.g. phenyl, o-chlorophenyl, p-methoxythiophenyl, 3'-phosphodiester of 5',2'-disubstituted nucleosides have been prepared, but they are however too labile during oligonucleotide synthesis (Reese 1978)).
- (iv) Phenylthio group. This is a base labile group introduced into oligonucleotide synthesis by Hata and Sekine (1974).

1.5.3 CONDENSATIONS (SYNTHESIS OF INTERNUCLEOTIDIC LINKAGE)

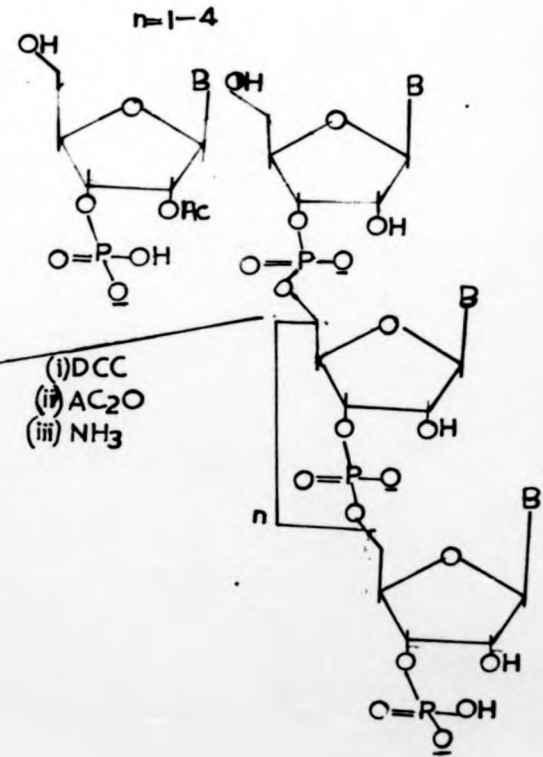
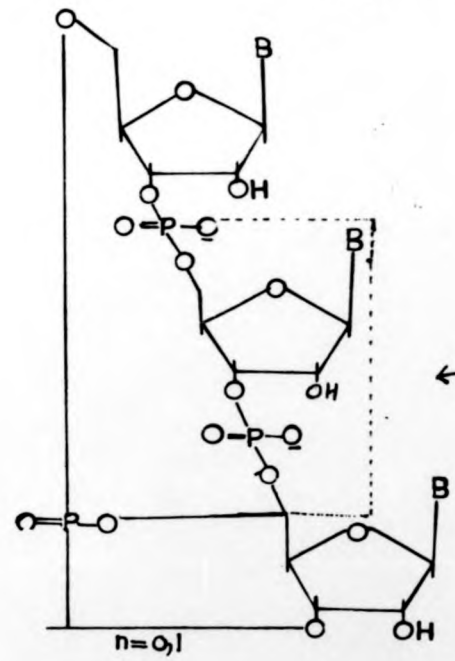
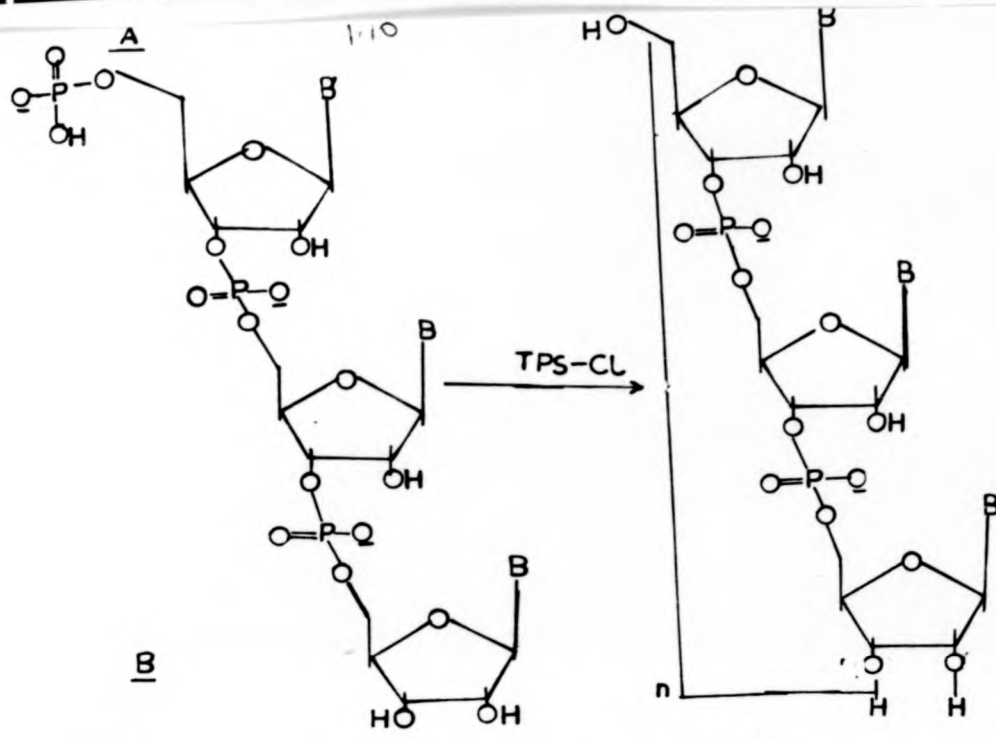
Formation of the internucleotidic bond is usually accomplished by reaction of a free alcohol of one nucleoside or nucleotide with the reactive phosphate of another nucleotide. Various approaches have been reported for making the phosphate molecule amenable to nucleophilic attack by hydroxy. These include the following:

(a) Non-specific Phosphodiester Linkage

This involves the formation of diribonucleoside monophosphate, when the 2'-hydroxy function is not masked in the nucleoside destined to be at the 5'-end. This results often in both 3'-5' and 2'-5' isomers, e.g. condensation of ribonucleoside 2',3'-cyclic phosphate with the

FIGURE 1.10 Chemical polymerisation

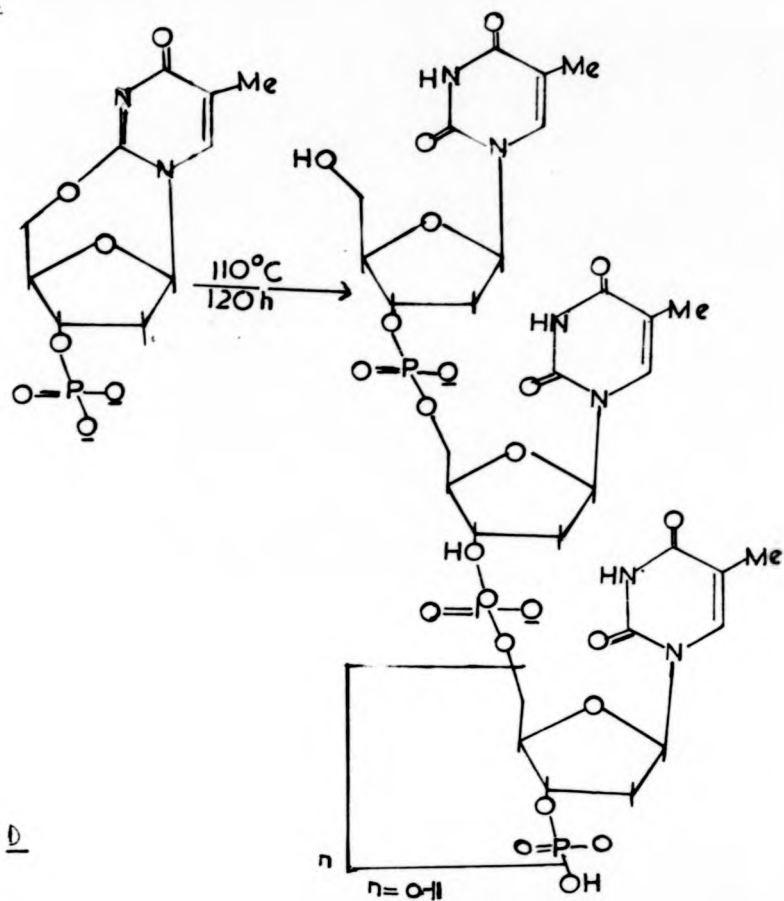
- (A) Block condensation,
- (B) Condensing agents mediated polymerisation,
- (C) Uncontrolled self-condensation,
- (D) Advantages of diester approach.



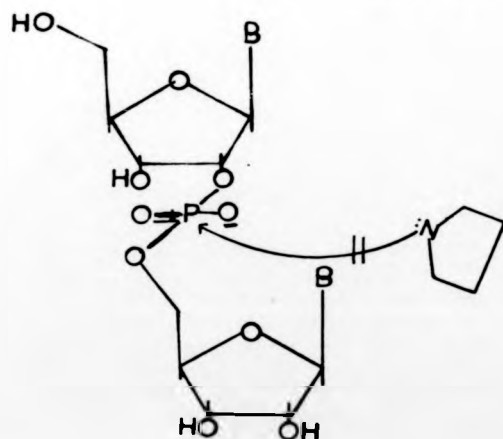
- (i) DCC
- (ii) AC₂O
- (iii) NH₃

isation,

C



D



2',3'-isopropylidene acetal of a nucleoside in the presence of diphenyl phosphorochloridate and tributylamine, this led to a mixture of 3'-5' and 2'-5' dinucleoside monophosphates.

The drawback of this approach is that at the end of condensation, the isomers must be separated before addition of another nucleoside.

(b) Chemical Polymerisation

This involves uncontrolled condensation which leads to oligonucleotides with repeating units which may either be a single nucleotide or a small chain of nucleotides. For ribonucleotides, the 2' (or 3') hydroxy is protected in order to yield 3'-5' (or 2'-5') internucleotide linkage. The products, which consist of a mixture of oligonucleotides of differing lengths, are purified by ion exchange chromatography. Examples of such polymerisation methods include the following.

(i) Polymerisation with condensing agents (e.g. TPSCl).

This method has been used to synthesise thymidylic, deoxyguanylic and uridylic acids, and oligonucleotides by Smith, et al. (1962) (Fig. 1.108).

(ii) Use of cyclonucleoside phosphates. Nagyvary and Nagpal (1972), employed O², 5'-cyclothymidine-3'-phosphate for the formation of oligomers through repeated condensations.

(iii) Kerr, et al. (1979) have used mixtures of 2',3'-adenylic acid to synthesis 2-5A (Section 1.6).

(c) Catalysed Polymerisations

Zinc ion, protic acids and 4-amino-5-imidazolecarboxamide, catalysed oligomerisation of adenosine, uridine and thymidine-5'-

triphosphate respectively. Pyrophosphate bond formation, and acidic cleavage of glycosyl bond in purine nucleotides are the major drawbacks. Pb^{2+} catalysed oligomerisation has been found to yield mainly 2'-5' linkages (Sawai and Orgel, 1975). This formed the basis of Sawai's approach to synthesis of 2-5A (Section 1.6) after the biological importance of the latter became significant.

(d) Block Condensation

Oligomerisation of small chain of nucleotides with unprotected 5'- or 3'-hydroxy and 3'- or 5'-phosphate ends allows the preparation of large oligomers containing more than one kind of nucleotide. Greater disparity in molecular weight between successive members than is the case in mononucleotide oligomerisation, facilitated the separation of product mixtures (Fig. 1.12) (Ikehara, *et al.*, 1979).

(e) Polymerisation on Templates

Poly(U) has been used as template for polymerisation of 5'AMP with itself, or with adenosine. The coupling took place in presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Similarly, self-condensation of guanosine 5'-phosphate and its coupling with guanosine was facilitated specifically by a polycytidylic acid template to give dinucleotide, pyrophosphate, and trinucleotide (Ikehara, *et al.*, 1979).

(f) Stepwise Synthesis in Solution

The non-specific oligomerisation procedures described above are useful, but they have limited use because of the following:

- (1) Oligonucleotides with specifically defined primary structure cannot be produced.
- (2) Efficiencies of self-condensations are often low.

(3) Oligonucleotides of varying lengths result, whose separations may be tedious.

(4) Pyrophosphates are often present.

All these problems are avoided when nucleotide residues are condensed in a stepwise manner. The advantages of this approach include the following:

- (a) Yields of individual condensations tend to be relatively high.
- (b) It is only by the stepwise approach that the oligonucleotide blocks may be prepared.
- (c) Most of the studies hitherto reported are designed for methodological development and are much more readily carried out at the di-, tri-, or tetranucleotide level.

However, the coupling yields tend to decrease with increasing chain length, and also it is often difficult to separate the product and the starting oligonucleotide, because of the relative similarity in their sizes. There are two types of condensation resulting in joining two nucleotide residues. In type I condensation, a 5'-nucleotide (Fig. 1.1d), is coupled with a free 3'-hydroxy function of properly protected nucleoside. The type II condensation involves the reversal of condensation I, i.e. a 3'-phosphate (Fig. 1.1c). is condensed with a 5'-hydroxy. In general, the type I condensations are most applicable in the less hindered 2'-deoxy derivatives, whereas type II condensations involving 3'-phosphate and 5'-primary alcohol groups are applicable to both ribo- and deoxy-ribonucleotides.

(i) Type I Condensation

An oligonucleotide chain of a single nucleoside bearing an

acid-labile monomethoxytrityl group or protected phosphate moiety at its 5'-end is lengthened in two steps (Fig. 1.1d). This strategy of building from the 5'-end to the other has an inherent disadvantage in that there occurs steric hindrance by the 2'-substituent to the approach of 5'-phosphate, (usually carrying a bulky activating group,) to the 3'-hydroxy functions, thus the use of this methodology has not progressed beyond the dinucleoside level.

(ii) Type II Condensation

Condensation between a 3'-nucleotide and the 5'-hydroxyl of a nucleoside represents the most common approach to synthesis of oligonucleotides (Fig. 1.1c). This is the approach employed in this thesis for 2-5A. This condensation has been useful in both diester and triester synthesis.

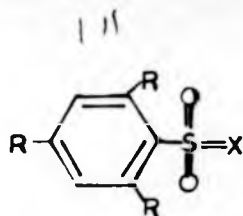
(a) Phosphodiester Method Basically, a 3'-nucleotide is added to 5'-hydroxy at the growing end. The protecting group at the 5'-end of the chain is released after each condensation under conditions to which 2'-hydroxy and 2',3'-cis-glycol blocking agents are insensitive. The synthetic scheme involving the acid-labile trityl protecting group was used by Lohrmann et al. (1966) for synthesis of all the 64 possible ribotri-nucleotides, this is the approach used in this work for synthesis of 2-5A (Fig. 1.7). This approach requires the isolation of intermediate compounds in neutral or weakly acidic media and at low temperature. Some of the difficulties have been mentioned in this review. In addition, there is difficulty in carrying out acylation of a 3'-nucleotide on a large scale,

and multistep procedures are often required for the synthesis of 2',3'-di-O-acyl nucleosides (or any other protected nucleoside). The later disadvantage was circumvented in this work by use of isopropylidene adenosine, which is base and acid stable enough for subsequent treatments in oligonucleotide synthesis. Despite the disadvantages, the approach has been successfully used by Lohrmann et al. (1966), Lohrmann and Khorana (1964) and now by us, to synthesise biologically important oligonucleotides. As mentioned earlier, one of the shortcomings of the diester approach is the time-consuming separation of the starting and the end product oligonucleotides, the yields are generally poor. Techniques such as ion exchange chromatography, thin-layer chromatography, and reverse phase chromatography have improved the isolation and characterisation of oligonucleotides.

(b) Triester Method Because of the above-mentioned problems, protection of the phosphodiester residues has been shown to be a successful solution to this problem. Other advantages of the triester approach have been mentioned in this review. The major problem in the triester method is that the rate of internucleotide bond formation is lowered by a factor of 10, hence the period of reaction is usually prolonged. A report by Catlin and Cramer (1973) has also suggested that the presence of a 5'-phosphate triester unexpectedly stabilised the 3'-O-acetyl group such that its removal caused some deprotection of amino bases. This sort of observation, coupled with the fact that greater chances of

- FIGURE 1.11
- (A) Arenesulphonyl derivatives for coupling phosphodiester intermediates.
 - (B) Tautomeric forms of triazole.
 - (C) Tetra- and disubstituted phosphates.

A



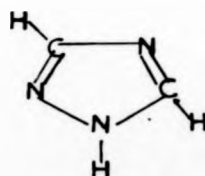
COMPOUND

← R →

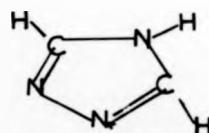
X

	ISOPROPYL	METHYL	H	
CL	TPS	MS	BS	
NI)))	
TET)))	
TRI)))	
IMI)))	

B

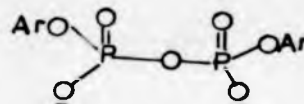
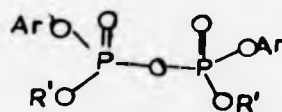


1H Form
(a)



4H Form
(b)

C



exchange exist because of the relatively electron poor centre of the phosphate moiety, thus encouraging the electron rich incoming coupling agent to react, this may limit the use of triester approach (Fig.1.10b) Despite these setbacks, the triester approach has been found useful in synthesis of tri- and tetra-nucleotides. Possible choice of phosphate protection groups have been listed earlier. Ideally, it would be highly desirable to combine the relatively high reaction rate of the diester approach with the convenience of isolation of the triester method.

1.5.4 CONDENSING AGENTS

This section is well documented in earlier reviews cited, and additional useful information relating to this topic and others discussed in this thesis are well documented by Hobbs (1980, in print) and Hutchinson (1979). In oligonucleotide synthesis, the key reaction is the joining together of two components consisting of a free phosphate function and another hydroxy without prior activation to form either a 3'-5' or 2'-5' internucleotide bond. The condensing agents show increasing complexity, and those commonly used are as follows.

(i) DCC.

(ii) Arenesulphonyl chlorides, imidazoles and triazoles.

See Fig.1.11.

The reagent, TPS-Cl, continues to find wide use. There is no doubt that it is an effective activating agent during the synthesis

of internucleotide bond in the diester and it is recommended for use during the second step in the phosphotriester approach. It is preferred to MS-Cl which reacts more slowly with the free 5'-hydroxy functions (also see page 103). However, the use of TPS-Cl often leads to darkening of reaction media, and sulphonation of the free 5'-hydroxyl group and relatively slower rate of reaction. The nature of intermediates produced in treating mono- and oligo-nucleotides with arenesulphonyl chlorides have been investigated by ^{31}P n.m.r. (Knorre and Zarytova, 1976). It was found that when arenesulphonyl chlorides are used, in the presence of internucleotidic phosphodiester groups, nucleotidic phosphate, or as activating agents in triester approach, trisubstituted pyrophosphates, monomeric metaphosphate (or its pyridinium adduct) or tetrasubstituted pyrophosphates are obtained respectively (Fig. 1.11c)

Thus, efforts have been concentrated on obtaining an activating agent which rapidly converts phosphate bearing nucleoside, into the intermediates without side reactions. This led to the use of the arenesulphonyl imidazoles, triazoles and recently tetrazoles. The imidazoles promote phosphorylation without darkening of the reaction media and without sulphonation of the free hydroxyl group. However, the reactions proceed very slowly. The triazoles promote phosphorylation more slowly than chlorides, but they are about three times faster than the imidazole derivatives. Narang, et al. (1972), have found that the tetrazole derivatives of the arenesulphonyls offer high yield and short reaction times. These reagents however, decompose on storage. Van Boom, et al. (1977), modified the TPS-imidazole into the

TPS-4-nitroimidazole, and found that it acts as a powerful agent in oligonucleotide synthesis. The latter reagent promotes phosphorylation more slowly than TPS-Cl in synthesis of dimers and trimers. However, reaction proceeded rapidly during block synthesis of higher molecular weight oligomers. The reduced rate of sulphonation of free hydroxys when substituted sulphonyl derivatives are used, is due to the steric hindrance caused by the presence of substituents in ortho positions of TPS, BS and MS. The structures of 1,2,4-triazole have recently been studied by Guimon, *et al.* (1980). It is found to exist in the tautomeric forms shown (Fig. 1.11B), and a predominates in solution whereas b predominates in the crystalline state.

In this project we evaluate the usefulness of some commonly used sulphonyl derivatives (Section 1.8.7) bringing about the formation of the phosphodiester linkage. Other less popular activating methods include using the following condensing agents:

- (1) Poly(3,5-diethylstyrene)sulphonyl chloride.
- (2) 2,2'-dipyridyl disulphide and triphenylphosphine.
- (3) Water soluble condensing agents, e.g. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

Note added: After the conclusion of our studies on the condensing agents, Ogilvie and Pon (1980) observed a similar trend in usefulness of the activating agents in triester synthesis. Our study was concerned mainly with diester synthesis.

Because of the above problems involved in the use of the arene-sulphonyl derivatives as coupling agents in internucleotide synthesis, and due to their being sensitive to moisture, some workers still hold

strongly to their once-useful DCC. This is an alternative method for activation of phosphoric acids using carbodiimides. This method can tolerate appreciable quantities of water. This is a useful method for the diester approach since protection of the extra acidic functions on the phosphomonoester is unnecessary, and condensation reactions take place at room temperature. It has been found that when DCC is used for diester synthesis, excess DCC reacts with the internucleotidic links, thus protecting them. The amount of DCC to be used for a condensation should therefore allow for this side-reaction. The hydration product, dicyclohexylurea, is highly soluble in most solvents and can easily be removed at the end of the synthesis.

Mode of Action of Condensing Agents

The mechanism of formation of phosphate esters by DCC or arenesulphonyl derivatives is still a matter of conjecture. There are two schools of thought.

- (1) ^{31}P n.m.r. spectroscopic evidence and polymeric studies have shown that treatment of nucleotides (ROPO_3^{2-}) with DCC and arenesulphonyl chlorides, leads to formation of nucleoside monomeric metaphosphate or its pyridinium adduct. This undoubtedly will be a powerful phosphorylating agent for anions, amines and alcohols. However, when arenesulphonyl chlorides are used in the presence of internucleotide phosphodiester groups, neither mono- nor trimetaphosphates were observed. Signals due to a complex mixture of products were observed, including those

due to the tetrasubstituted symmetrical pyrophosphate obtained when TPS-Cl is used as activating agent in the second step of the triester approach (Fig. 1.12). This latter finding seems to support Khorana's alternative mechanism namely:

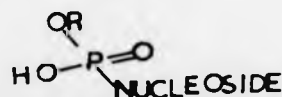
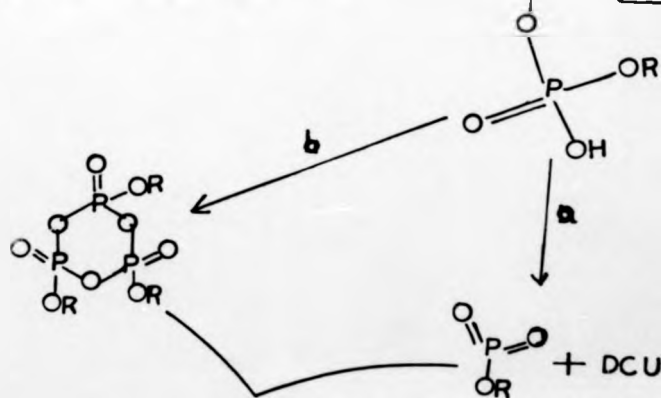
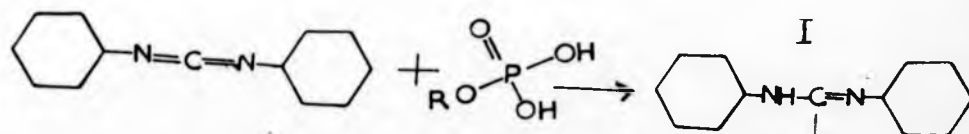
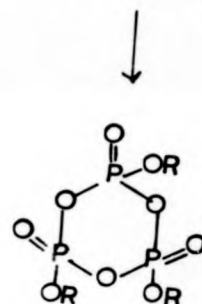
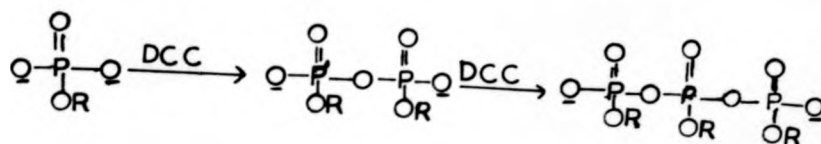
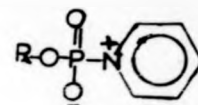
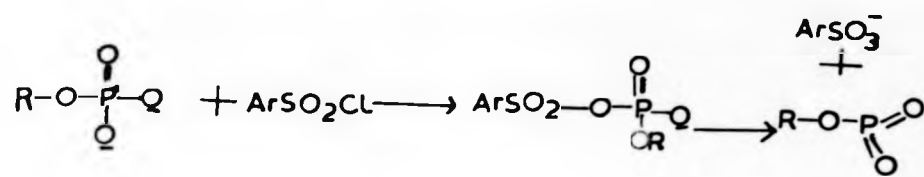
- (2) Trinucleoside trimetaphosphates are formed as a result of stepwise reaction of nucleotide with DCC in anhydrous solvents. This is presumably by trimerisation of monomeric metaphosphates, as shown in Fig. 1.12.

Knorre and Zarytova (1976), concluded that when arenesulphonyl chlorides are used as condensing reagents, monomeric nucleotide derivative (i.e. nucleotide metaphosphate or its pyridinium adduct) is the highly reactive intermediate. In the presence of phosphodiester groups in the nucleoside or nucleotide component, the significantly less reactive derivatives with trisubstituted pyrophosphoryl residues are formed both with arenesulphonyl chloride and DCC. These conclusions were made on the basis of data obtained by pulsed n.m.r. spectroscopy.

It is generally agreed that when DCC reacts with a phosphoric acid, an imidoyl phosphate I (Fig. 1.12) is formed initially, but whether this breaks down to monomeric metaphosphate (pathway a) or whether it reacts with more phosphoric acid to give polyphosphate esters (pathway b) is still controversial. Carbodiimide-mediated condensations unlike sulphonyl halide-mediated condensations, can take place in the presence of appreciable quantities of water. This suggests that the highly reactive monomeric metaphosphates may not be intermediate in these reactions. Despite the controversy over the exact mechanism of DCC action, ease of use has resulted in DCC being applied to synthesis of many phosphodiester and oligonucleotides. Along with

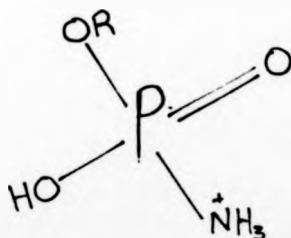
FIGURE 1.12 Mode of action of condensing agents.

1.12 MODE OF ACTION OF CONDENSING AGENTS.



other coupling agents, we evaluated its usefulness.

Nucleoside polyphosphates can be prepared using DCC, but when two different phosphoric acids are brought into reaction with carbodiimide, random formation of the two symmetrical and one asymmetrical products occurs, thus leading to low yields. In addition, in the presence of orthophosphoric acid, polymeric products can be formed, thus reducing yield. Thus, DCC has been replaced by various phosphoramidates (e.g. diesters of phosphoramidic acid) (Fig. below). Phosphoroimidazolates have been found to be more reactive than simple phosphoramidates, thus N,N'-carbonylbisimidazole reacts vigorously with phosphomonoesters (e.g. 5'AMP) to give phosphoroimidazolates. This has been found useful for preparation of nucleotide polyphosphates. This reagent was used in this project to synthesise the triphosphorylated 2-5A from the phosphomonoesterified 2-5A (Section 1.8.8).



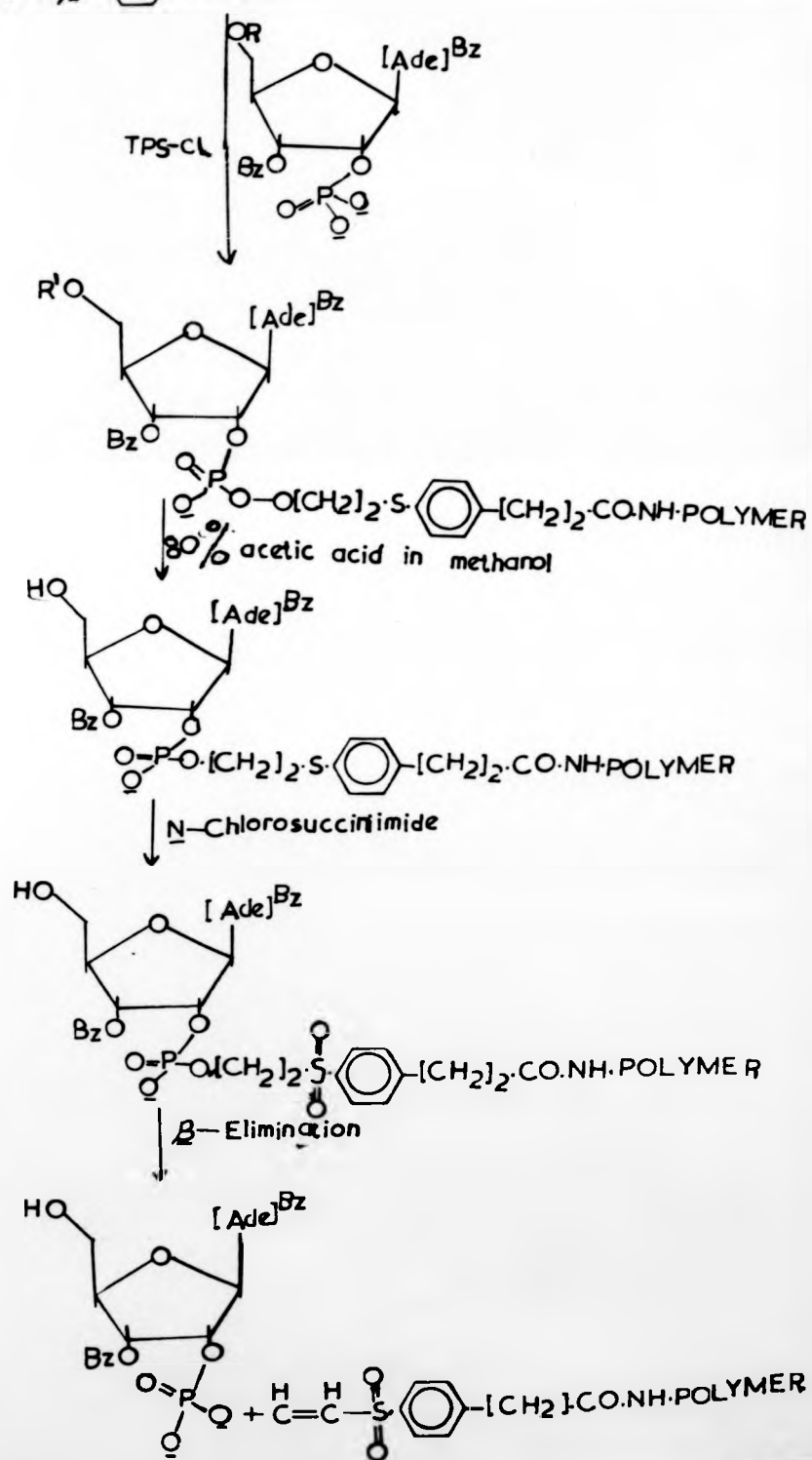
(A) Synthesis of Core 2-5A(i) Solid Phase Synthesis: Diester Approach

The discovery of 2-5A was followed by the publication of various chemical syntheses of the compound. As mentioned before, the yields are often unreported (or otherwise, they are extremely low), in agreement with the problems of synthesis of ribonucleotides as discussed above.

Solid phase synthesis of the core was reported by Markham, et al. (1979), with a coupling yield of 10%. The solid phase synthesis allows the nucleoside or nucleotides to be joined covalently to a polymer support and subsequent nucleoside units are added stepwise. This allowed major purification to be attained simply by filtering the polymer-supported oligonucleotide and washing away other soluble by-products and excess reagents. Since the separation of oligonucleotides from other reagents at the end of coupling in solution is usually laborious and time consuming, solid phase synthesis should be more powerful, faster and high yielding than synthesis in solution. At the end of the synthesis the polymer may conveniently be removed to yield the product. The method has been fully investigated for the diester synthesis only. However the use of the method may be extended to triester method, and especially since most polymers in use are non-polar in nature, the existence of the non-ionic phosphotriester moieties in proximity to the non-polar support may be of immense advantage during the synthesis of oligonucleotides.

FIGURE 1.13 One complete cycle of nucleotide addition to resin during the synthesis of 2-5A on a polymer support.

Markham etal (1979).

$$\text{OH}(\text{CH}_2)_{32}\text{S}\cdot\text{C}_6\text{H}_4\text{-(CH}_2)_2\text{-CO-NH-POLYMER}$$


For the solid phase synthesis of 2-5A, Markham, et al. (1979) chose the diester method of Khorana. It is interesting to compare the ease and otherwise of this method with our synthetic approach (see Section 1.8.3). Both follow similar routes except that Markham and co-workers have used solid phase while we have chosen solution synthesis of oligonucleotides. The authors however emphasised that their synthesis was an initial feasibility study of the application of the polyamide solid phase method to oligonucleotide synthesis. The scheme is shown in Fig. 1.13. Similar to our approach, the 5'-O-trityl N⁶,3'-O-dibenzoyl adenosine 2'-phosphate ~~were~~ synthesised and these units were repeatedly condensed with each other. One complete cycle of the nucleotide addition to the resin involves:

- (a) Reaction of functionalised resin with 6-fold excess of the 5'-O-trityl N⁶,3'-O-dibenzoyl adenosine phosphate.
- (b) Deactivation of activated phosphodiester bonds by treatment with pyridine/water.
- (c) Resin drying with pyridine solution of phenyl isocyanate.
- (d) Terminal deprotection with acetic acid/methanol.

Three such cycles were carried out using bench-top apparatus, with 5'-O-trityl N⁶,3'-O-dibenzoyl adenosine 2'-phosphate as the nucleotide derivatives, to give the protected trinucleotides. The cycles showed coupling yields of 63% and 30% at dimer and trimer stages respectively. These represent substantial increases in yields over the synthesis of 2-5A in solution using the same diester approach with similar building blocks.

An important variation of this work from the synthetic approach

described in this thesis involves the use of protected nucleotides as the terminal and non-terminal unit during the chain extension. The advantage of this choice is clearly seen in Section 1.8.3, where we, like previous workers have encountered difficulties with synthesis of protected nucleoside derivatives. This contrasts with the relative ease of synthesis of protected nucleotides (Section 1.8.5). Thus the removal of this major bottleneck of oligonucleotide synthesis, coupled with the advantages of solid phase in contrast to solution synthesis, enabled Markham and collaborators to obtain higher overall yields of 2-5A than any other published procedure up to date. Apart from the use of polyamide solid-phase, all other methods used are standard solution techniques employed for synthesis of oligoadenylates. The C.D. spectra, and other characteristics of the synthetic oligoadenylate are similar to those of the natural product. The technique appears to have no apparent limitation for the synthesis of oligonucleotides.

(ii) Stepwise Synthesis in Solution: Triester Approach

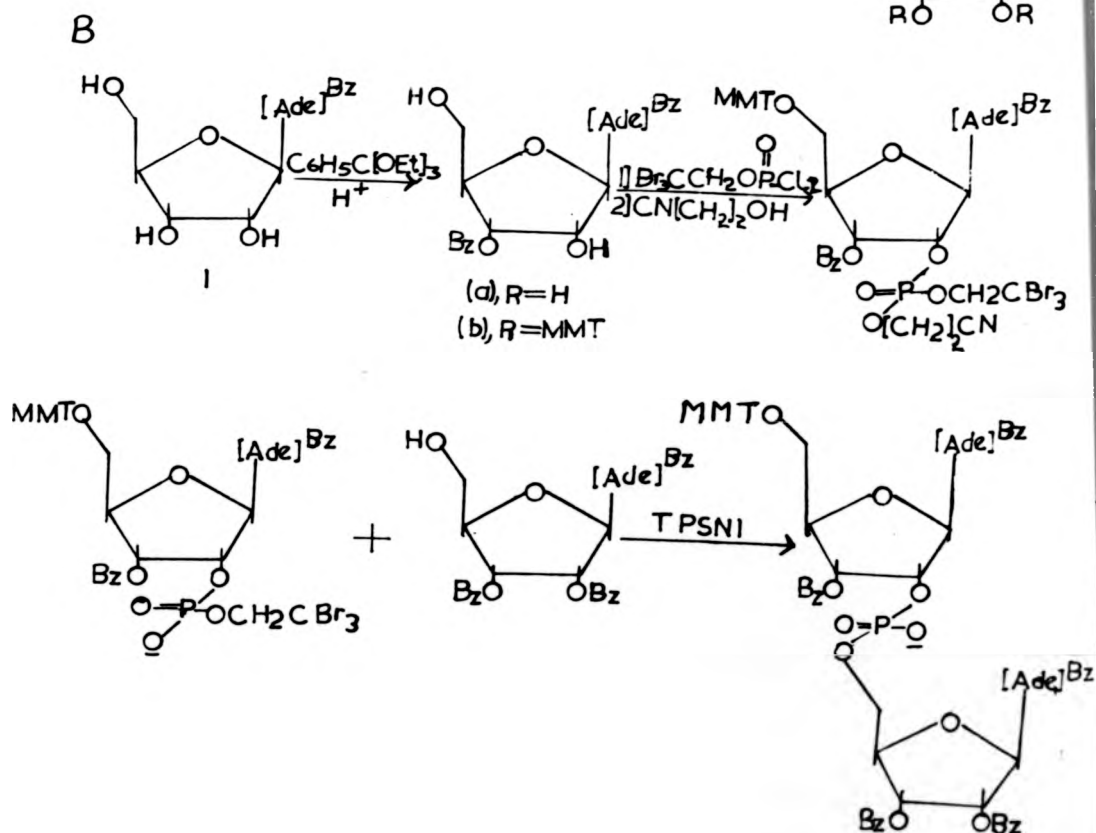
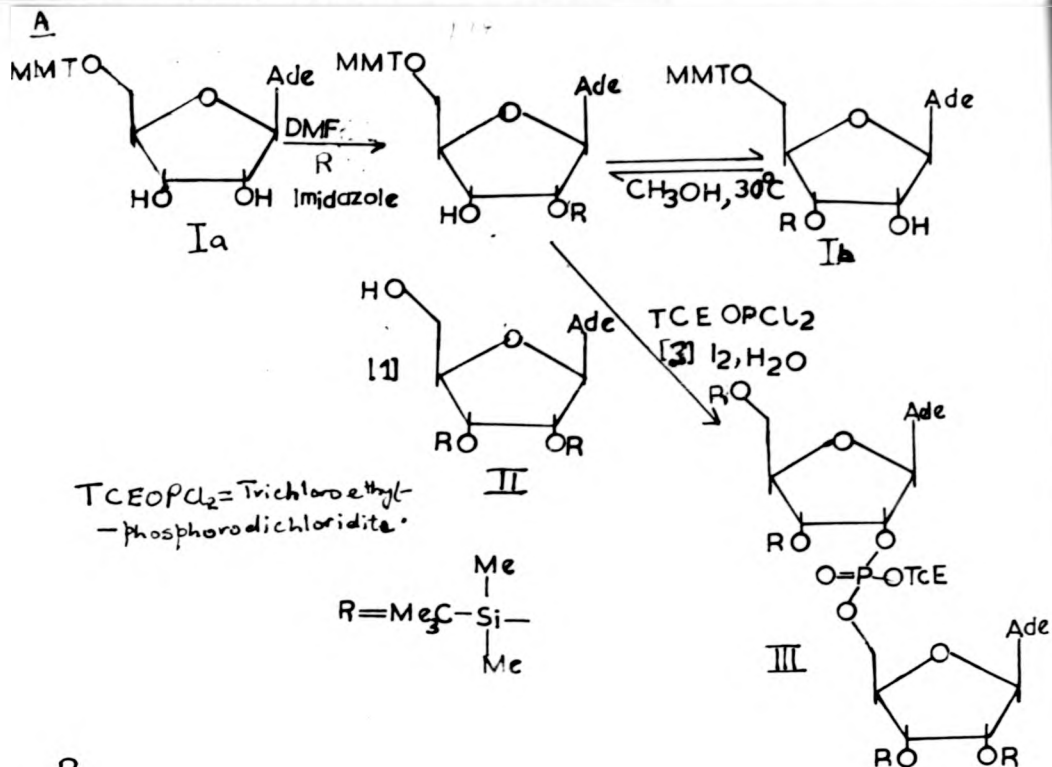
Ogilvie and Theriault (1979) reported the synthesis of core oligonucleotide using alkyl silyl protecting groups which they had previously developed for synthesis of oligonucleotides. In this method, starting from 5'-MMT adenosine Ia (Fig. 1.14A), alkylsilyl protecting groups were used to synthesise protected ribonucleosides Ib and II which were directly linked together by the chlorophosphite procedure to form ribonucleotides III (Fig. 1.14A). Treatment of Ib with III gave the protected trinucleotide. The method appears to be simple and fast, and yields obtained are reasonably good, being ca. 66% for the dinucleotide stage. The major setback of the procedure lies in the fact that while the

FIGURE 1.14 (A) Stepwise Synthesis of 2-5A in solution.

Ogilvie and Theriault (1979)

(B) Synthesis of core trimer by stepwise
synthesis in solution.

Engels and Kramer (1979)



silyl groups are stable in most solvents, isomerisation between the 2'- and 3'-silyl nucleosides occurs in methanol with the 2'-silyl derivative predominating by about 10% over the 3'-derivative. This led to the formation of two diastereoisomers of the protected dinucleoside. They were separated by t.l.c. Similar diastereomers were obtained at the trinucleoside stage. Although the diastereomers were subsequently separated, the possibility of the existence of representative proportions of each diastereomer in the preparations cannot be ruled out. It was also our experience that separation of diastereomers of silyl adenosine was not as easy as was claimed by the authors. Taken together the synthesis provides a rapid route to synthesis of core 2-5A, and one should think that if the final step is combined with phosphodiesterase reaction, the product should be reasonably pure 2-5A.

(iii) Stepwise Synthesis in Solution: Triester

The second method for synthesis of core 2-5A in solution was performed by the triester approach. Engels and Krahmer (1979) chose to use N⁶-benzoyl adenosine (I) (Fig. 1.44) as the starting material. It has been found (see Section 1.8.4) that prior benzylation of the amino of adenosine renders the synthesis of the fully protected nucleoside much easier. Departing from the use of popular benzoylating agents (e.g. benzoyl chloride), the authors 3'-benzoylated with orthobenzoic triethyl ester. Tritylation of the 5'-hydroxy position gave the partially protected nucleoside which was 2'-phosphorylated with (tribromoethyl) phosphoric dichloride in triazole and N-propylimidazole followed by 3-hydroxypropionitrile, gave, after chromatography on silica gel (CHCl₃/MeOH 25:1), the fully protected nucleotide triester in 82% yield, probably the first time when such a high yield would be obtained for synthesis of fully protected nucleotide from the nucleoside. Conversion of the

nucleotide triester to the diester and condensation of the latter in the presence of TPS nitroimidazole with tribenzoyl adenosine gave the dinucleoside phosphate in 90% yield (yield apparently referring to coupling step). Major disadvantages of the approach have been mentioned before and include the following.

- (i) Problems involved with synthesis of tribenzoyl adenosine.
- (ii) Possibility of obtaining a low yield of N⁶,3'-O-dibenzoyl adenosine due to lack of protection at the 2'- and 5'-hydroxys, and the attendant problem of obtaining the pure desired product. About 2% 3'-5' linked dimer was formed, and clearly the amount of the unwanted product will increase with chain length.

However, the method appears to be rapid and high yielding overall. The products obtained have identical characteristics with data published in the literature. The synthetic approach makes use of some relatively new protecting groups (e.g. orthobenzoic triethyl ester) and deblocking procedures (e.g. debenzoylation with sec. butylamine in methanol).

(B) Synthesis of 2-5A

All the synthetic approaches so far discussed, and all known up to date have used the phosphotriester approach. The major differences have been in the use of different combinations of blocking group.

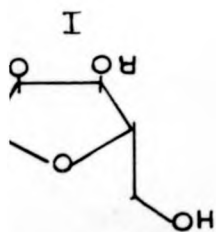
Synthesis of the triphosphorylated core as opposed to synthesis of the core alone discussed so far, has also been accomplished by a number of workers. den Hartog, et al. (1979) have synthesised the triphosphorylated core starting with two properly protected building blocks, i.e. the non-terminal units I and II (similar to the type already discussed in this thesis). Two phosphorylating agents have been employed a and b (Fig. 1.15).

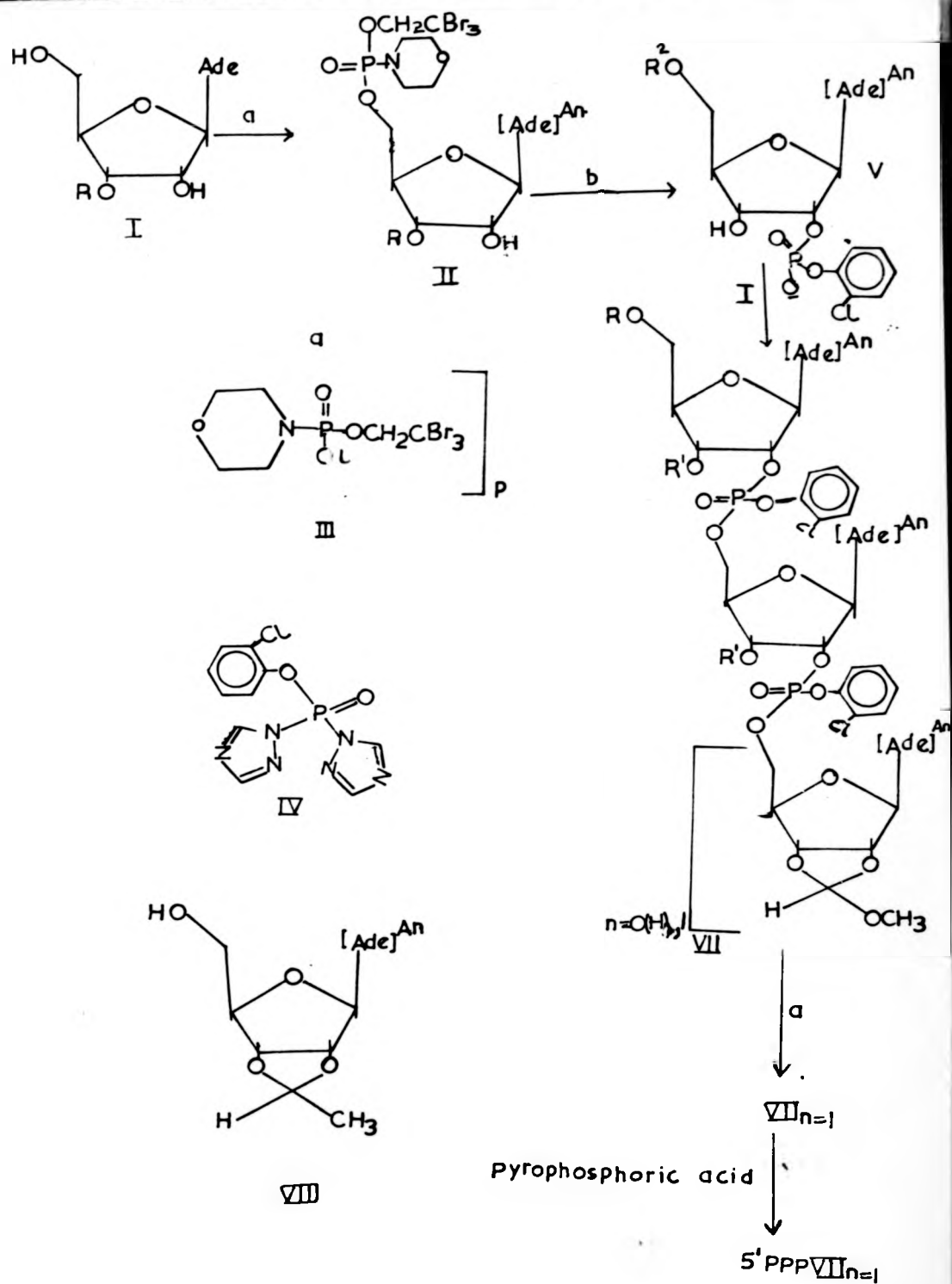
The approach was to synthesize the 5'-phosphorylated nucleoside II initially, which was subsequently re-phosphorylated using the second phosphorylating agent. Condensation of V with the non-terminal unit I gave the dinucleoside phosphate VII. The dimer VII was phosphorylated and condensed to terminal unit VIII to give the protected 5'-phosphorylated core; the 5'-phosphate protecting group was removed and the product converted to the triphosphorylated core via pyrophosphoric acid.

The advantage of this technique involves the use of two condensing reagents, the first one, which has specificity for a primary hydroxy group, a monofunctional phosphorylating agent, allowing the phosphorylation of the 5'-position. The second phosphorylating agent, a bifunctional phosphorylating agent was used for the introduction of the 2'-5' internucleotide linkages. The method is very specific for synthesis of the 2'-5' linked oligonucleotide. It however appears to be cumbersome and since no yields were quoted it is likely to have been very low overall. The other advantage

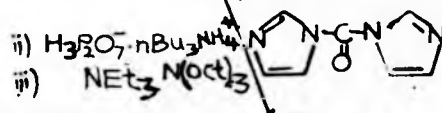
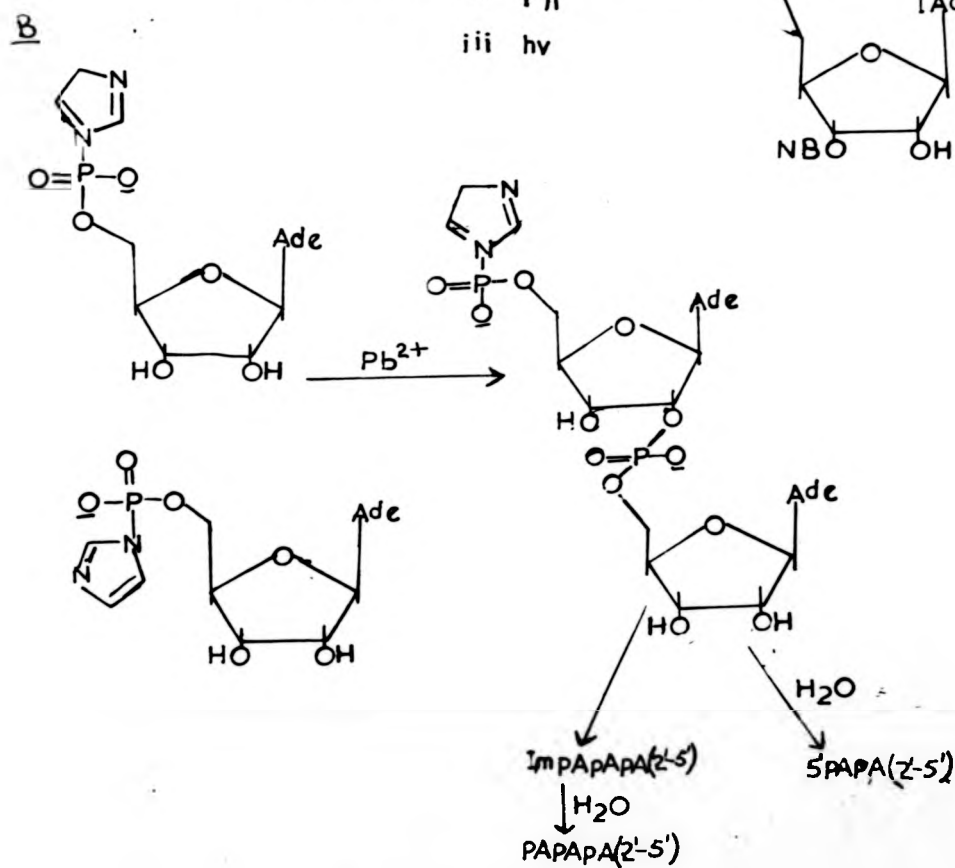
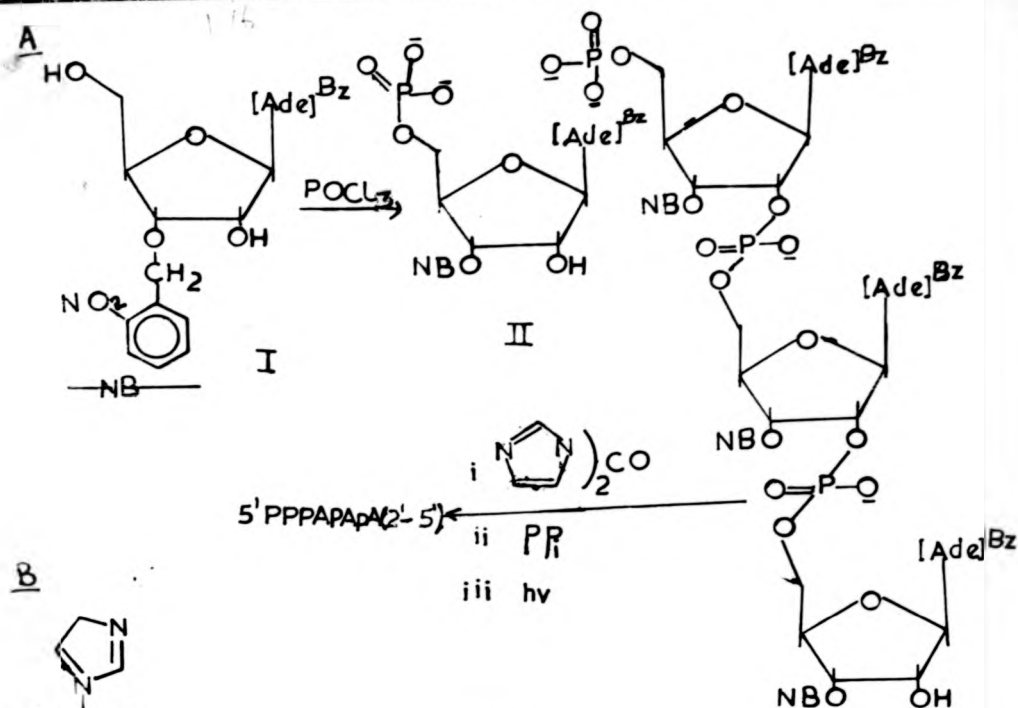
OH

FIGURE 1.15 Synthesis of 2-5A: Phosphotriester approach.
den Hartog et al. (1979)





- FIGURE 1.16
- (A) Use of N-benzoyl-3'-O(o-nitrobenzoyl) adenosine as starting material for synthesis of 2-5A. Ikehara et al. (1979).
- (B) Synthesis of 2-5A by chemical polymerisation. Sawai et al. (1979).



NEt₃ = Triethylamine
 N(Oct)₃ = Tri-n-octylamine
 5'PPPAPAPA(2'-5')

involves protection of the terminal as a diol system with an acid-labile group (similar to the one we used, see Section 1.8), which was stable to subsequent treatment during the synthesis. The monofunctional and bifunctional phosphorylating agents used are shown as (a) and (b) respectively.

Condensation Method

Ikehara (1979) chose to use N⁶-benzoyl-3'-O(o-nitrobenzyl) adenosine I (Fig. 1.16A) as the main building block for their synthesis of the triphosphorylated core (Fig. 1.16A). I was phosphorylated with phosphorus oxychloride. The phosphorylated product II was condensed using DCC. After 15 days at room temperature, the reaction mixture was worked up and the trimer was obtained in 7.7% yield. The product was phosphorylated using the method of Hoard and Ott (1965) which gave a yield of 40% from the core. The triphosphate yield obtained is comparable to that obtained by den Hartog (1979) discussed above, and the low yield of the core is consistent with general problems of oligonucleotide synthesis. In addition, as discussed under synthesis of oligonucleotides via condensation, the yields are very low, and the approach is beset with problems already discussed. In this synthesis, higher oligomers predominated, (ca. 14%), major products likely being unwanted side products due to lack of protection on the phosphate and uncontrolled condensation. The separation of product is likely to be another major problem in this approach, since oligomers will be synthesised in series. It will be advantageous, if this technique could be adapted such

that condensation could be performed much faster (e.g. by use of TPS-tetrazole). The final step involves the removal of the o-nitro-benzyl protecting group by u.v. light.

Chemical Polymerisation: Pb^{2+} : Template

Earlier, Sawai and Orgel (1975) have reported that Zn^{2+} ion promotes the synthesis of oligoadenylates in neutral aqueous solutions, and substantial amounts of oligoadenylates with 2'-5' linkage were obtained. Sawai, (1976), Weimann et al. (1968), Orgel and Lohrmann (1974), have also reported that in the presence of template, oligoadenylates can be synthesised through Pb^{2+} catalysis, and that in the presence of Pb^{2+} , the product is predominantly 2'-5' linked oligoadenylate. Taken together, these two studies appear to suggest that Pb^{2+} can catalyse the synthesis of oligoadenylates, in presence of template and Pb^{2+} mainly 3'-5' linked oligomers are formed; the presence of template but absence of Pb^{2+} leads to synthesis of 2'-5' linked oligoadenylates in > 90%. and that in the absence of template but presence of Pb^{2+} , the major product again is 2'-5' linked oligoadenylate. This last discovery led Sawai, et al. (1979) to polymerise adenosine 5'-phosphorimidazolid (ImpA) in aqueous solution using lead nitrate as catalyst to synthesise $ImPA2'p5'A2'p5'A$. Hydrolysis of the product with water, produced $pA2'p5'A2'p5'A$. This latter product was phosphorylated with the method of Hoard and Ott (1976).

Chemical polymerisation with/without lead ion is hindered by most of the problems already discussed under condensation, and so in the case of chemical polymerisation here which involves the use of ImpA which is not protected at the secondary hydroxy functions. A number of side products are likely to emerge whose purification will lead to low isolated yield of desired product. The isolated yield of the diphosphate ImpApA(2'-5') and ImpApApA(2'-5') was quoted to be 24% and 9% respectively, and the authors mentioned the identification of other oligoadenylates. The second stage of the reaction ImpApA(2'-5') \rightarrow ImpApAp(2'-5') is sensitive to water which could lead to synthesis of 5'pA2'p5'A. This latter compound if present in aqueous solution, will increase the chances of possible side products. The yield was low, but the synthetic route was simple, less complicated and less time consuming (Fig. 1.16B).

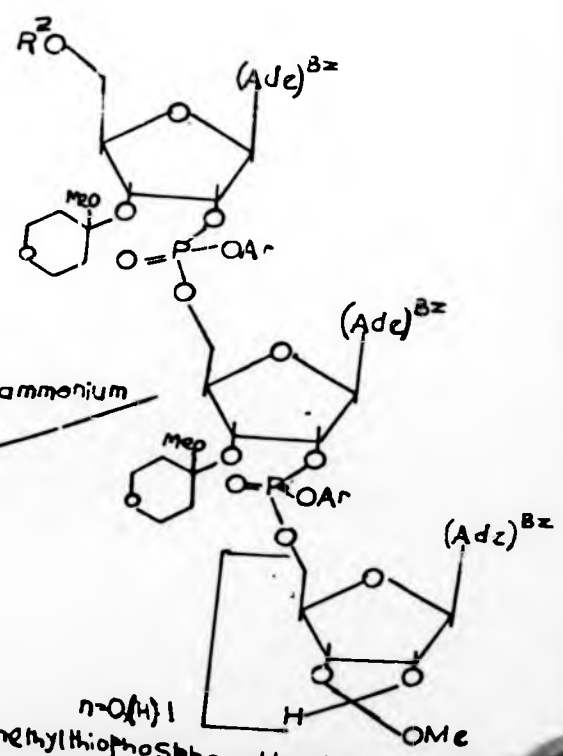
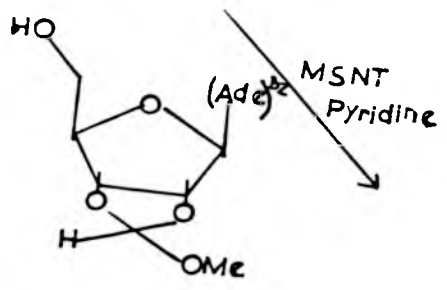
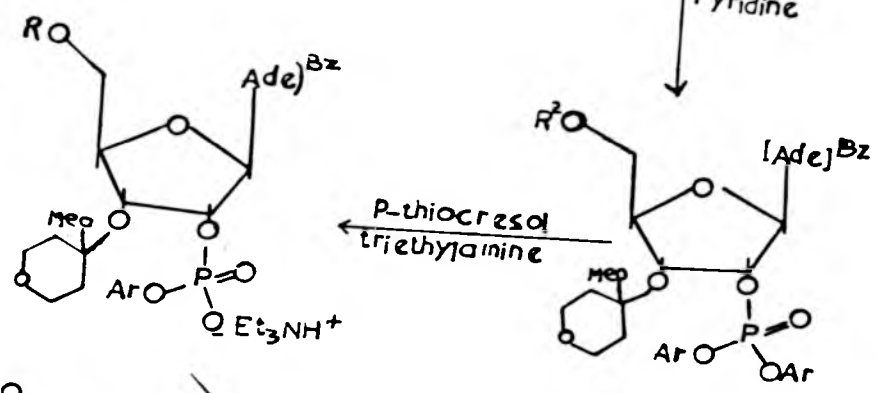
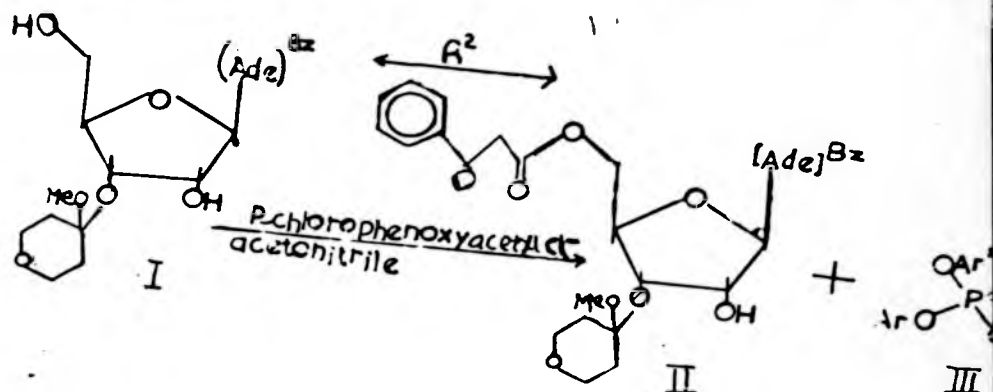
Triester Approach

Jones and Reese (1979) used the triester approach for the synthesis of 2-5A. They chose to start with 3'-O-methoxytetrahydropyranyl-6-N-benzoyladenine I (Fig. 1.17). The approach is similar to that employed by den Hartog (1979) already discussed. Similar 3'-protecting groups were used in both cases, and similar bifunctional phosphorylating agents were used for the introduction of the 2'-5' linkage. The apparent difference lies in the fact that while den Hartog chose to introduce the 5'-protected phosphate

into the building block, Jones and Reese (1979) masked the 5'-hydroxyl with an alkali labile protecting group (p-chlorophenoxyacetyl) throughout the synthesis of the core. This was subsequently released at the last stage of core synthesis. The partially protected core was treated with pixyl chloride, which is specific for the 5'-hydroxy group, and similar to the novel 5'-protecting group we developed (see Section 1.8.4). This allowed the selective deblocking of the core, to yield the 2'-5' linked oligoadenylate. This approach offers immense advantage in that it reduces the chance of isomerisation or cleavage of the 2'-5' linked oligoadenylate, and it reduces the chance of 5'-5' linked oligonucleotide, and other side products. All these side products are often obtained during the synthesis of oligoadenylates by previously mentioned approaches, especially during the process of deblocking. From evidence gathered in all approaches to synthesis of 2-5A, the syntheses of the core trimer are the limiting steps, and the yield in this method, quoted as 75%, was expectedly good (assuming that the protected nucleosides are shelf items!).

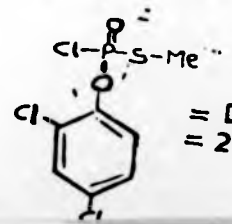
The authors used the synthesis of the 2-5A to demonstrate the versatility and usefulness of a novel 5'-phosphorylating agent, (0-2,4-dichlorophenyl-S-methylthiophosphorochloridate) introduced in 1978, to convert the core into the corresponding 5'-O-(2,4-dichlorophenyl) S-methylphosphorothioate, through the reaction of this novel phosphorylating agent with the partially protected core (Fig. 1.17). Hoard and Ott's method yielded the 5'-O-triphosphorylated oligomer in average overall yield. Thus, in most stages of the synthesis, the yields were good, and the synthesis of the triphosphate derivative of the

- FIGURE 1.17
- I = 3'-O-methoxytetrahydropyranyl-6-N-benzoyladenosine
 - II = 5'-O-p-chlorophenoxyacetyl-3'-O-methoxytetrahydro-
pyranyl-6-N-benzoyladenosine
 - III = 2-chlorophenyl 4-nitrophenyl phosphorochloridate
(Ar = 2-ClC₆H₄; Ar' = 2-O₂NC₆H₄)

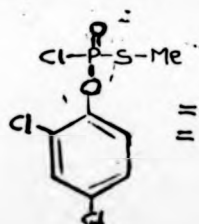
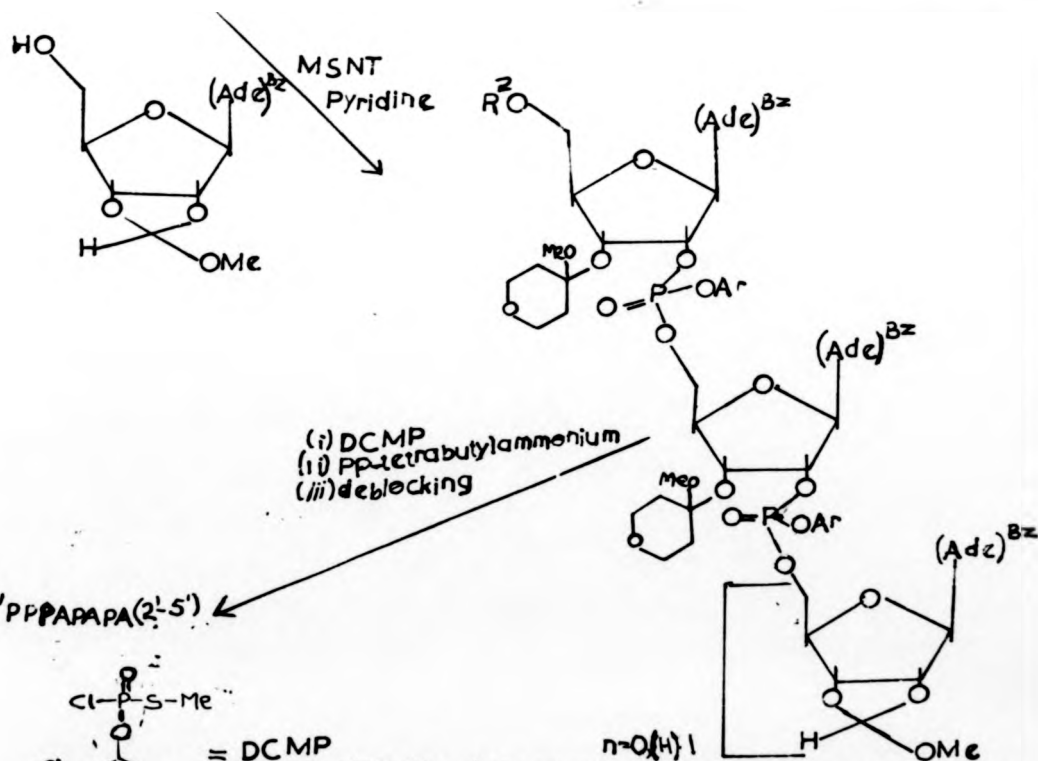
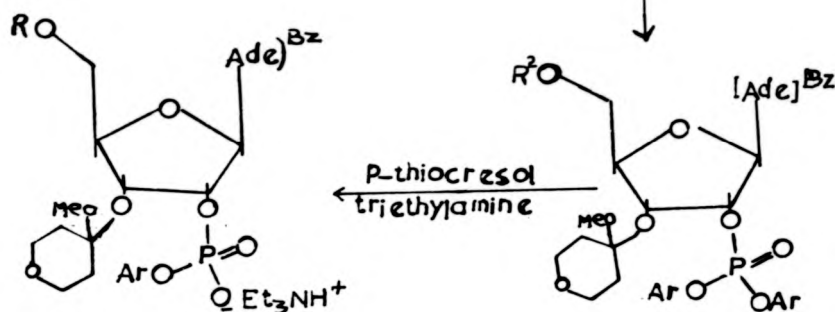
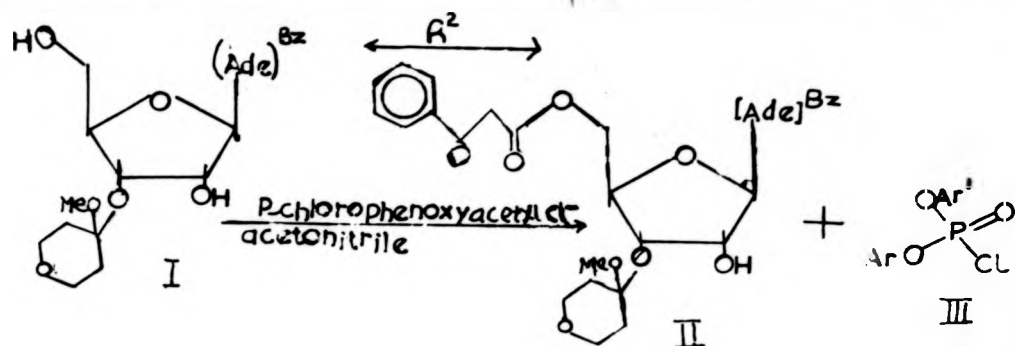


(i) DCMP
 (ii) p-pentabutylammonium
 (iii) deblocking

5'PPAPAPA(2'-5')



= 2,4-dichlorophenyl S-methylthiophosphorochloridate
 $n=0(4)1$



$= DCMP$
 $= 2,4\text{-dichlorophenyl S-methylthiophosphorochloridate.}$

$n=O(H)1$

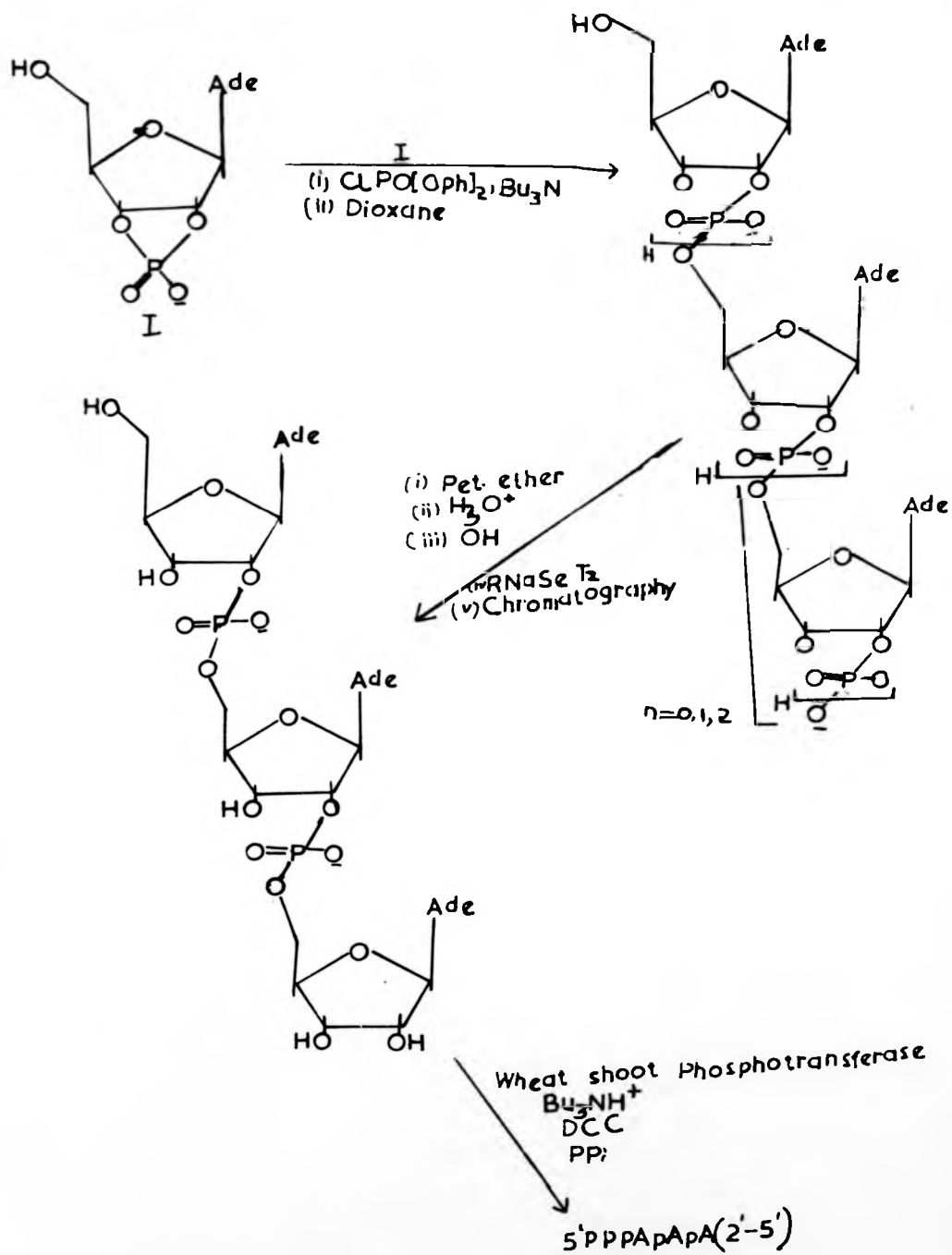
monophosphate gave about average yield. This contrasts with the methods described earlier, where the chain extensions were low yielding steps but the terminal phosphorylation gave fairly good yields, just as was obtained by Jones and Reese (1979). If problems involving the synthesis of protected nucleosides are circumvented, this approach appears to be one of the best so far used for synthesis of 2-5A.

Finally, for all stages of condensation, 1-mesitylene sulphonyl (3-nitro-1,2,4-triazole) (see Section 1.8.8) was employed, this differs from den Hartog's 1979 method where TPS nitroimidazole was used for condensation. Both reagents have been reputed to be good for triester synthesis.

Non-Specific Phosphodiester Linkage

The pioneers of the works on 2-5A (Kerr, et al. 1976), used the methods of Michelson (1959), for the chemical synthesis and characterisation of 2-5A. The starting material was a mixture of 2':3'-AMP, with no hydroxy protecting groups, and the method is close to random condensation. This non-specific synthesis of phosphodiester linkage involves the condensation of the ribonucleoside 2',3'-cyclic phosphate I (Fig. 1.18) together in the presence of diphenylchlorophosphate and tri-n-decylamine. This led to production of mixtures of 3'-5' and 2'-5' dinucleoside monophosphates. One of the major drawbacks of this approach is that at the end of condensation the isomers must be separated prior to the addition of another nucleotide. However, Martin et al. (1979) chose to repeat the condensation of a third nucleotide without prior separation of the

FIGURE 1.18 Synthesis of 2-5A via non-specific phosphodiester linkage.
Martin et al 1979.



phodiester linkage.

dinucleosides. Realising that a high proportion of their products will likely be A3'p5'A3'p5'A and A2'p5'A3'p5'A, the final product was digested with T2 RNase to yield the 2-5A oligoadenylate only. The yield was expectedly low at 8%. The advantage of the method lies in its simplicity, and the fact that several blocking and deblocking steps are unnecessary. It may be useful for the synthesis of dinucleoside phosphate, its use for higher oligomer being very doubtful. The method inherited all the disadvantages of the diester approach in addition to problems of non specific phosphodiester linkages (see page 28) and the number of possible products is too numerous for anyone to be certain that extensive purification will yield the final pure product. The use of 2',3'-isopropylidene acetal of adenosine as the terminal nucleotide, as originally suggested by Michelson (1959), might have improved the yield, although the terminally formed 2',3'-cyclic phosphate may serve the same purpose, but this will be less efficient compared to the use of terminal isopropylidene adenosine. This is because the terminal cyclic phosphate will cleave to yield a 2'(3')-phosphate mixture, in the presence of the diphenylphosphorochloridate and the amines used for condensation given in the reaction scheme. Clearly the method may not be very useful for synthesis of specifically linked oligoadenylate. The authors did mention that the method was crude.

The oligoadenylate was phosphorylated by either our enzymic method (see Chapter 2) or polynucleotide kinase. The wheat shoot enzyme used was a crude preparation, and interestingly the yield of the 5'-monophosphate was about 40% (see Chapter 2). The use of the crude extracts will pose additional complications during this

synthesis, since other enzymic activities (2',3'-phosphotransferase and phosphodiesterases and 5'-diesterases) may be present. It may also make the isolation of the product very difficult, since in addition to unreacted side products, protein may be eluted along with the oligonucleotide. We have found that sodium acetate of ionic strength close to the ammonium bicarbonate buffer used by these authors, precipitated some proteins from a crude extract of wheat shoot phosphotransferase (Ademola and Hutchinson, 1980). Importantly, however, the 5'-monophosphorylated oligonucleotide was isolated and converted to the triphosphate using DCC and pyrophosphoric acid. The yield was expectedly poor at 5%. The problems and advantages associated with the use of DCC have been discussed above. The final products have spectroscopic, analytical and n.m.r. properties, similar to the natural product. Significantly, the natural and chemically synthesised materials have identical specific biological activity in inhibition of cell free protein synthesis.

2-5A has been a subject of intensive research recently, but no direct evidence is available for its involvement in interferon action in virus-infected cells. As mentioned earlier experiments have shown that two enzymes (2-5A synthetase and protein kinase) which are induced in response to interferon, by treatment of interferon-treated CFS with dsRNA, have been assumed to play a vital part in the action of interferon (Nilsen and Baglioni, 1979). In addition, the 2-5A synthetase is present in the absence of interferon treatment and is also capable of using important metabolites as substrates, these suggest wider significance for the 2-5A system in the control of cell growth and development.

The 2-5A has attracted the attention of organic chemists trying to synthesise the biologically active molecule, identical to the natural compounds (e.g. Markham, *et al.* 1979). Whereas there are numerous publications about the biological activities of the naturally isolated 2-5A, and researches have been progressive in the chemical synthesis of 2-5A and its analogues, most of these studies have not indicated any biological activity of the chemically synthesised product. The situation is quite understandable, since establishment of identical structure of the chemically synthesised product with natural product, does not necessarily mean obtaining a correspondingly similar biological activity, especially because a biological system may be sensitive to any chemical inadvertently introduced via the chemically synthesised 2-5A. However, to realise

the optimal functions and uses of 2-5A, the chemical synthesis of the molecule and its analogues, and the effects of these products on wider ranges of cells must be studied. Screening and comparative studies of the actions of these chemically synthesised products with actions associated with interferon, may give clues to the role of the 2-5A in mechanism of action of interferon. Such studies may also lead to the isolation of a more biologically potent 2-5A than the natural product. The above objectives will reduce the problems associated with isolation, toxicity, and uses of interferon, if indeed it is finally established that the action of interferon is mediated by 2-5A. Studies tend to indicate that this may actually be the case. 2-5A systems have been said to be responsible for the control of cell growth and development, and its analogues may therefore be of potential medicinal use. This is supported by the fact that the biologically synthesised material contains, in addition to the triphosphorylated trimer, variable and smaller amounts of higher oligomers. The biological significance of these oligomers remains to be determined (Kerr and Brown, 1976). Apart from the direct potential uses of chemically synthesised 2-5A and analogues, they can also serve as tools for the understanding of other roles of 2-5A in cells treated with 2-5A dependent endonuclease. It is known that accumulation of viral mRNA is inhibited in these cells, but it is not clear whether this inhibition is due to impairment of transcription, translation or cleavage of viral template.

The mechanism by which interferon makes cells resistant to virus infection and its other actions have evaded attempts at

unifying concepts for many years. It seems possible that interferon-induced mechanisms have diversified to allow the host cell multiple methods to manage various types of viruses. Of all these diverse modifications, the phenomena of interferon-induced virus restrictions via translation-inhibition have gained universal acceptance. The translation-inhibition theory itself comprises of many possibilities. Thus, while the molecular basis of the multiplicity of effects is yet to be elucidated, significant advances have come from the idea that 2-5A plays a vital role in the action of interferon. The potential therapeutic use of interferon has been mentioned earlier. Because of these reasons coupled with the fact that the 2-5A may be involved in other aspects of cellular metabolism, we were encouraged to study the 2-5A and some of its biological properties. We chose to synthesise the 2-5A by chemical methods, study the phosphorylation of the core by enzymic and chemical methods, and finally study the effects of the synthetic oligonucleotide in MG63 cells. The effects of either natural or synthetic 2-5A on these cells have never been examined before. In the study of effects of 2-5A on cells, we intend to look into the nature of the protein synthesis inhibitory action of the 2-5A and as an additional means of confirming the nature of, and biological activity of, the synthetic product. We hope to correlate the observed biological activity with that obtained using natural 2-5A as our contribution to confirmation of the nature and biological activity of 2-5A. We hope the study will contribute to the understanding of the mode of action of interferon and 2-5A and possible wider roles for 2-5A.

Identification of the necessary conditions for the synthesis of such oligonucleotides followed by necessary characterisation of the oligomer in terms of physical, chemical, and sensitivity to enzymatic activity, are essential, in order to assess their likely usefulness in a biological system. In particular, one of the major problems in this work arose from the use of diester approach for the synthetic work (see section 1.8.6). While the technique proves faster than triester approach, in the isolation of the products, it suffers from the extremely low yield obtained, extensive side products and therefore the need for extensive purification. These may be due in part to the presence of unprotected phosphodiester bond, and therefore liability to side reaction. These have previously been reported whenever the diester approach is used for the synthesis of oligonucleotides (see page 28). It will be useful to be able to retain the speed and simplicity of this technique and improve the yield and decrease the side product formation in the synthesis of oligonucleotides. A considerable length of time was therefore spent looking into the possibility of enhancing the yield of individual steps involved in the synthesis, and reaction conditions involved. Time was also spent on studying the best protective groups which may lead to achievement of the above objective, and we also tried to introduce a novel 5'-hydroxy protecting group, code named MPDA (Fig. 1.8).

Once the core oligonucleotide had been synthesised and characterised, the introduction of phosphorus at the free 5'-hydroxy was undertaken by the use of wheat shoot phosphotransferase enzyme. The carrot enzyme has been successfully used to

phosphorylate a series of nucleosides, and other unrelated compounds of biological interest (Brungraber and Chargaff, 1967). The specific requirement appears to be the presence of the primary hydroxy group (Giziewicz and Shugar, 1975). These authors have also suggested that unpurified wheat shoot fraction may have broader specificity in terms of the sugar moiety requirements. We therefore chose the wheat shoot enzyme for phosphorylation of the core 2-5A, taking advantage of its specific requirements of primary hydroxy function, and allowed broader base specificity.

There are many chemical phosphorylation techniques adaptable for nucleosides, and the chemical conversion of the nucleoside-5'-monophosphates to di- and triphosphates can be accomplished in high yield using phosphomorpholidates or related intermediate (Moffatt and Khorana, 1961). On the other hand, chemical phosphorylation of nucleoside at the 5'-position often yields low products and can be accompanied by the production of large amounts of inorganic phosphates making purification of the nucleotide difficult (Yoshikawa, *et al.* 1967). There are two additional problems. The 2-5A core to be phosphorylated contains adenine base which may give unwanted side products with phosphorylating agents. Secondly, techniques for phosphorylating nucleosides may prove disastrous if employed for oligonucleotides, because of the presence of phosphodiester bonds which may react with any agent in the solution, and thus lead to possible isomerisation and/or bond breakage. In fact, up to date, the only available chemical method for suitable phosphorylation of oligonucleotide, proceeds with isomerisation of 2'-5' and 3'-5' phosphodiester bonds,

leading to a mixture of products, often impossible to separate completely. Clearly this situation is not satisfactory in the synthesis of 2'-5' specifically linked oligomer. The use of chemical methods for phosphorylation of the unprotected oligonucleotide has to await further developments. However, the use of enzyme as mentioned above, offers the possibility of site specific phosphorylation of oligonucleotide, and we found the wheat shoot enzyme to be particularly useful for phosphorylation of unnatural and atypical oligonucleotide A2'p5'A2'p5'A.

The difficulty in the synthesis of the oligonucleotide not only calls for an agent that will specifically phosphorylate the 5'-hydroxy of the oligonucleotide, for subsequent introduction of pyrophosphate bond, but it also calls for an efficient phosphorylation technique. Thus, the establishment of optimum phosphorylation of the oligonucleotide would necessarily entail extensive purification of the wheat shoot enzyme. To improve the yield of the purified enzyme, we studied its properties and suggest a new phosphate donor in the form of 2,4-dinitrophenylphosphate, which we found led to an improvement in the phosphorylated product.

Phosphotransferase has a great potential for the specific phosphorylation of unusual oligonucleotides, polynucleotides and sugar bearing compounds of biological interest with primary hydroxyl groups. However, several other factors limit the use of the purified enzyme on a large scale.

The most important of these are high cost, time of isolation, and difficulty of recovering an enzyme from the reaction mixture. We therefore looked into the possibility of using the enzyme for a

large scale synthesis. The discovery and use of immobilised enzyme derivatives which retain their specificities and catalytic activities and can be used repeatedly, offered us this possibility. We therefore developed optimum conditions for the immobilisation of the wheat shoot phosphotransferase, studied the kinetic properties of the immobilised enzyme, and used it in a reactor for the repeated synthesis of 5'-phosphorylated 2-5A.

EXPERIMENTAL

1.8 MATERIALS AND METHODS

MATERIALS

- (a) RNase A type XII-A (E.C.3.1.4.22), RNase T₁ (E.C.3.1.4.8) RNase T₂ (E.C.3.1.4.23), yeast hexokinase (E.C.2.7.1.1), and snake venom phosphodiesterase from Crotalus adamanteus (E.C. 3.1.4.1) were obtained from Sigma.
- (b) Bacterial alkaline phosphatase (E.C.3.1.3.1) was obtained from Boehringer.
- (c) Thin layer chromatography plates were Kieselgel 60 HF₂₅₄ and were a product of E. Merck, Darmstadt. Paper chromatography was performed on Whatman papers.
- (d) All other reagents were of analytical grade. Reagent grade pyridine was distilled and dried over calcium hydride or molecular sieve (4A) (Linde Co.) for a week.

GENERAL METHODS

- (a) Chromatography was performed by upward development for the plates and downward development for paper, using the following solvent systems:

A.	2-propanol-concentrated ammonia-water	7:1:2 v/v
B.	1-propanol-concentrated ammonia-water	55:10:35 v/v
C.	1-propanol-concentrated ammonia-water	6:3:1 v/v
D.	Isobutyric acid-concentrated ammonia-water	66:1:3 v/v
E.	Sat. ammonium sulphate-1 M sodium acetate- isopropanol	80:18:2 v/v
F.	Chloroform:methanol	8:2 v/v

Ultraviolet absorbing material was visualised by fluorescence under 254 nm light in the case of plates. Nucleotides on paper were visualised using the method of Cashion, et al. (1977).

Relative mobilities of unprotected nucleotides are expressed with respect to those of 2'-AMP 1.00 and adenosine (0.00).

- (b) The trityl-containing compounds were made visible on paper chromatograms after spraying the chromatograms with the perchloric acid spray (10%), and warming in an oven for 30 minutes. The compounds containing mono-p-methoxytrityl group appeared yellowish-orange, and those containing di-p-methoxytrityl group appeared orange-red. Alternatively, trityl derivatives are detected by means of ceric sulphate spray by heating the sprayed chromatogram at 300°-400° to give a yellowish-orange colour (Neilson and Werstiuk, 1971).

All protected derivatives were run on t.l.c. using silica gel HF₂₅₄, solvent F.

- (c) Phosphorus was assayed by the method of Chen, et al. (1956).
- (d) The abbreviation O.D₂₆₀ refers to the extinction of a nucleotidic solution at neutral pH at 260 nm in 1 ml of a solution using a 1-cm light path quartz cell.
- (e) Samples of natural pppA2'p5'A2'p5'A, A2'p5'A2'p5'A, A2'p5'A were generously supplied by Kerr, I. M., and these served as standards during characterisation studies.
- (f) Adenosine-2'-monophosphate was purified on a Dowex-2 (Cl⁻ form 2 cm x 35 cm) column eluting with gradient of ammonium chloride, (0.1-0.4 M). Each fraction was made alkaline (pH 9.0) with ammonium hydroxide and was readsorbed on Amberlite IRA 400 (OH⁻ form, 0.7 cm x 10 cm), elution was first with ammonium chloride (0.01 M, 70 ml.) and the major adenosine 2'-phosphate peak was eluted with minimum of ammonium chloride (0.1 M, 28 ml.). The nucleotide was concentrated and precipitated as the calcium salt.

Rf's. in Solvent E

3'AMP	0.36
2'AMP	0.38
5'AMP	0.28

- (g) Oligonucleotides and nucleotides were separated by the method of Asteriadis, et al. (1976).

TABLE 1

Nucleotides	V ^a (ml) with solvent system:	A	B	C
A2'p5'A2'p5'A		50	52	
A2'p5'A		30	40	
Ap2'		28	35	
Ap3'		20	25	
pp2'p5'A2'p5'A		55	-	180
ppA2'p5'A2'p5'A		-	-	192
pppA2'p5'A2'p5'A		-	-	250

^aChromatographic retention volumes obtained with columns (100 x 0.4cm) of Dowex 1-X2 (-400 mesh) ion-exchange resin by elution at 5 ml/hr.

^AElution with 500 mls of 40% ethanol containing a linear gradient of 0-0.4 M NH₄Cl (pH 8.0).

^BElution with 500 ml of 35% ethanol containing a linear gradient of 0-0.4 M triethylammonium bicarbonate buffer (TEAB), pH 8.0

^CElution with 500 ml of TEAB (pH 7.5).

In addition to other techniques used for identifying synthetic oligonucleotides and the products of their digestion, the above method of Asteriadis, et al. (1976), was found useful in identification of such materials, and when co-chromatographed in the presence of suitable markers, well resolved peaks were obtained.

(h) Segregation of oligonucleotide according to charge and size

was performed by the method of Tener (1968) using appropriate markers. For analytical runs, specific quantities of the markers and sample applied were noted, and where a sample co-migrated with a marker, the former was identifiable from the apparent increase in O.D. of the respective marker. Size and charges were identified by location and point of elution of fraction containing the sample to be identified. Fig. 1.19 shows the fractions and charges of samples.

- (i) Spleen phosphodiesterase activity was determined by the method of Brownlee (1972).
- (j) Periodate oxidation and β -elimination were performed by the methods of Schmidt (1968). Products were applied to Whatman No. 1 paper chromatogram and developed with solvent D.
- (k) Snake venom phosphodiesterase activity was determined by the method of Brownlee (1972).
- (l) Bacterial alkaline phosphatase activity was checked according to the method of Kerr, et al. (1978).
- (m) Pancreatic ribonuclease activity was determined by the method of Kerr, et al. (ibid).
- (n) Alkaline hydrolysis was performed by the method of Markham et al. (1979).
- (o) Micrococcal nuclease digestion was performed by the method of Rose (1975) and Brownlee (1972)
- (p) Adenine:phosphate ratio:
Adenine contents of oligonucleotide and nucleotide samples were determined by measuring the O.D. 260 of material (1 μ g) dissolved

in sodium cacodylate buffer (1 M, pH 8.5, 1.0 ml).

In all occasions the products were characterised by the following methods:

- (a) Paper chromatography, Whatman No. 1, solvents A-E. Followed by elution of different spots with 0.01 M HCl and O.D. plotted.
- (b) Analytical technique described under Section g above.
- (c) PEI cellulose chromatography in solvents A, B, C (Martin, et al. 1979).
- (d) DEAE Sephadex A-25 chromatography, in 50-400 mM sodium chloride in 20 mM tris-HCl (pH 7.6) - containing 7 M urea (Kerr and Brown, 1978).

1.8.3 SYNTHESIS OF FULLY PROTECTED NUCLEOSIDE DERIVATIVES

1.8.3.1 SYNTHESIS OF 5'-PROTECTED NUCLEOSIDES

5'-Trityl Adenosine

This was prepared by the method of Smith et al. (1962), except that trityl chloride was re-crystallised. Trityl adenosine was crystallised by subsequent heating in boiling pyridine (150 ml) and addition of ethanol (3.0 ml) yielded trityl adenosine as yellowish crystals at 0°. These were collected, washed in the ethanol and dried, yield 9.2 g. The mother liquor contains more trityl adenosine, and N,5'-O-ditrityl adenosine. The 5'-trityl

adenosine was freed of pyridine by heating twice in 200 ml portion of boiling water, on cooling, the 5'-trityl adenosine separates out.

Spectra characteristics are as follows:

λ_{\max} (ethanol) 260.5 nm, λ_{\min} 232 nm, Literature, Smith *et al.* (1962). λ_{\max} 260 nm, λ_{\min} 231 nm. Melting point: 250-255° (Lit. 253°). Homogeneous by solvent system F.

R_f (Tetrahydrofuran) = 0.6. 5'-Dimethoxytrityl adenosine: This was prepared as described for 5'-trityl adenosine except that dry dimethyl formamide with a trace of pyridine was used. The product was chromatographed on a column of silica gel by flash chromatography with a chloroform: Methanol (0-5%) gradient. The product was precipitated from ethyl acetate: cyclohexane containing a trace of pyridine. Yield was 35%. λ_{\max} (ethanol) 260 nm, λ_{\min} 232 nm. Literature, (5'-O-MMT-adenosine) Smith *et al.* (1962). λ_{\max} 260 nm, λ_{\min} 233 nm. Melting point: 201-205° (Lit. Mp. not given).

5'-O-Monomethoxytrityl Adenosine

The method of Lohrmann and Khorana (1964) was employed for the synthesis of this derivative, except that the product was chromatographed on a column of silica gel, and eluted with chloroform:methanol (2:1). Yield was 30%. The sample was recrystallised from ethyl acetate: cyclohexane. Spectral characteristics: λ_{\max} (ethanol) 260 nm, λ_{\min} 232 nm. Lit. (Lohrmann and Khorana, 1964). λ_{\max} (ethanol) 260 nm. λ_{\min} 233 nm. Melting point: 190-192° (Lit. Lohrmann and Khorana, 1964, 189-191°).

5'-O-Pixyladenosine

Pixyl chloride was supplied by Khandelwal, G. The adenosine derivative was prepared by modifying the method of Reese and

Chattopadhyaya (1978). The product was recrystallised from ethanol to give 70% of 5'-pixyladenosine. Melting point 190-194°. Lit. M.pt. = 194°. λ_{\max} (ethanol) 260 nm. λ_{\min} 232 nm.

5'-MPDA Adenosine

This was prepared following the procedure employed for synthesis of 5'-pixyladenosine, except that failure to obtain a crystalline derivative led to purification by silica gel using flash chromatography, and the column was eluted with gradient of chloroform:methanol (0-5%). Elution with chloroform alone gave a product, presumably N⁶, 5'-O-di-(MPDA)-adenosine. Subsequent elution with chloroform containing 2% methanol gave 40% (in O.D. units) of the 5'-O-MPDA-adenosine. Attempts to crystallise the product failed. Continued elution with chloroform containing 5% methanol followed by methanol alone gave only traces of the material. The amorphous powder melted at about 198°. Spectra properties: λ_{\max} (ethanol) broad maximum 260-265 nm. The spectral characteristics are close to those expected for the composite spectrum of MPDA chloride and adenosine mass spectrum: m/e, M⁺ = 591 (B + H)⁺ = 135. (MPDA + ribose + H⁺) = 446. The derivative ran as a single spot on t.l.c. (system F). Treating the derivative with acid (acetic acid 80% (Section 1.8.9)), gave only adenosine as the nucleoside.

MPDA -Chloride Synthesis

10, 10, Dimethylantrone

The lithium salt of anthrone was prepared by a modified method of Curtin, *et al.* (1960). The product was subsequently heated with methyl iodide and t-butylalcohol at 150° for 24 hours. Methyl iodide was distilled off, and the residue was digested with diethyl ether

and acidic product extracted with Claisen's alkali (350 g of potassium hydroxide in 250 g of demineralised water and diluted with absolute methanol to make the volume 1 litre). Removal of the ether from the neutral fraction yielded MPDA (4.2 g, 60%). The product was chromatographed on alumina and gave 3.1 g (50%).

Spectra characteristics: λ_{\max} (ethanol) 270 nm (Lit. 270 nm). ν_{\max} (Nujol) 1665 cm^{-1} (strong) (Lit. 1665 cm^{-1}). Melting point: 97-99° (Lit. 96.5-98°).

MPDA Chloride

In a 2-litre, three necked flask, fitted with a mechanical stirrer, a 50-ml dropping funnel, and a reflux condenser protected from atmospheric moisture (calcium chloride tube), were placed magnesium turnings (0.067 g, 0.036 g -atom) prewashed with ether and dried at about 15 mm Hg. Iodine (2 mg) was added, and the flask was warmed briefly with a free flame, and the flask was allowed to cool.

The magnesium turnings were covered with ether (8 ml) and p-bromoanisole (2-3 drops), was added with stirring. As soon as the reaction began, a mixture of p-bromoanisole (0.0352 mole) and dry ether (12 ml, predried by distillation over P_2O_5 and stored over sodium) was gradually added, with constant stirring. The mixture was heated under reflux with stirring, on a steam bath for 1 hour. The bath was removed, and freshly prepared dimethyl anthrone (4 g) was added at such a rate as to keep the mixture boiling. When the addition was completed, the mixture was heated under reflux, with stirring on a steam bath for 1 hour and kept at room temperature overnight. A solution of

and acidic product extracted with Claisen's alkali (350 g of potassium hydroxide in 250 g of demineralised water and diluted with absolute methanol to make the volume 1 litre). Removal of the ether from the neutral fraction yielded MPDA (4.2 g, 60%). The product was chromatographed on alumina and gave 3.1 g (50%).

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ammonium chloride (4.6 g) in water (10 ml) was added at such a rate which caused gentle boiling. The mixture was then steam-distilled until the distillate was clear. The distillation residue was cooled, and extracted with two portions of ether (2 x 10 ml) and the extracts were combined, dried over magnesium sulphate, filtered, and evaporated to dryness. The residue was dissolved in cyclohexane (16 ml), the solution was treated with thionyl chloride (4 ml) and the latter solution was heated under reflux with exclusion of atmospheric moisture (calcium chloride tube), on a steam bath for 15 min. The solution was evaporated to dryness and the residue was dissolved in a mixture of cyclohexane (4 ml) and acetyl chloride (0.4 ml) by boiling briefly. The resulting solution was kept at +2° overnight, and the MPDA was deposited. The product was rapidly filtered off with suction, washed with cyclohexane and dried in a vacuum desiccator over potassium hydroxide.

Melting point = 80°.

Tetrabenzoyladosine

This was synthesised via two routes.

(a) The first route modified the method of Smith et al. (1962) whereby the following derivatives were synthesised:

- (i) 5'-O-trityl-N¹,N⁶,2',3'-O-tetrabenzoyladosine,
- (ii) 5'-O-DMT-N¹,N⁶,2',3'-O-tetrabenzoyladosine,
- (iii) 5'-O-pixyl-N¹,N⁶,2',3'-O-tetrabenzoyladosine and
- (iv) 5'-O-MPDA-N¹,N⁶,2',3'-O-tetrabenzoyladosine.

Collectively, these are called 5'-O-x-N¹,N⁶,2',3'-O-tetrabenzoyladosine.

In each case the product was extracted with ethyl acetate, and washed thrice with sodium bicarbonate solution, and then washed twice with water. After being dried over

sodium sulphate, the ethyl acetate solution was evaporated to dryness. The residue was dissolved in hot 90% ethanol, and the solution in each case deposited the product. The product obtained in the foregoing experiment was in each case dissolved in glacial acetic acid (5 ml) and diluted with water (1 ml). After the time required for 5'-deprotection in each case (see experiments on acidic hydrolysis of derivatives) solid sodium bicarbonate was added until evolution of the carbon dioxide ceased. Water (5 ml) was added and the mixture extracted with ethyl acetate. After two washings with water, the extract was dried over sodium sulphate and then evaporated to dryness. The residue was dissolved in benzene and each solution gave the crystalline tetrabenzoate yields: (i) 25%, (ii) 28%, (iii) 30%, (iv) 29%.

Spectral characteristics are as follows.

λ_{max} (ethanol) 230 nm, 262 nm (Sh), 280 nm. Lit. (Khorana, 1962). λ_{max} 230 nm, 260 nm (Sh), 280 nm. Mass Spectrum: (B + H) = 135. Melting point: 178-180° (Lit. 179-181°). Homogenous by t.l.c. solvent F.

(b) An alternative approach to synthesis of tetrabenzoyl adenosine, was that of Schaller, et al. (1963). The procedure is similar to that described above except that adenosine was fully benzoylated with benzoylchloride at 50° for 5 hours. Subsequent work-up and the isolation of the product is as described in (a) above. The gum was dissolved directly in a mixture of ethyl alcohol (15 ml) and pyridine (10 ml), and was treated with a mixture of sodium hydroxide (20 ml, 2 N), and ethanol (20 ml) at room temperature for 5 min.

Excess pyridinium Dowex-50 was added to remove sodium ions, the washings were evaporated with frequent addition of pyridine and the product dissolved in pyridine (50 ml). The solution was added dropwise to ether (2 litres), and the resulting precipitate of N-benzoyl adenosine was collected by centrifugation. The product was washed with ether and dried.

The N-benzoyladenosine so obtained in the foregoing experiment, was converted to the 5'-tritylated derivatives, by the previously described procedures, except that N-benzoyladenosine dissolved better in the reaction medium (pyridine in dimethylformamide). Yields (shown below) showed slight improvement over method (a) except that as was the case in route (a), this treatment (b) failed to yield crystalline derivatives of 5'MPDA, N-benzoyladenosine.

Yields: (i)-(iv) referred to above. (i) 30%, (ii) 35%, (iii) 30%, (iv) 30%.

Spectra characteristics for N-benzoyladenosine: λ_{\max} (ethanol) 300 nm, 265 nm, 230 nm. λ_{\min} 290 nm, 250 nm, 210 nm. Lit. Schaller, *et al.* 1963). λ_{\max} 302 nm, 263 nm, 231 nm, λ_{\min} 295 nm, 250 nm, 213 nm. Spectral characteristics of tetrabenzoyladenosine are similar to those obtained via route (a).

Finally, benzylation of nucleoside either via route (a) or (b) was performed according to Holy and Sounček (1971) using benzoyl cyanide instead of benzoyl chloride. Apart from the poisonous properties of the side products, higher yields (50%) were obtained when the former benzoylating agent was used in each case.

1.8.4 SYNTHESIS OF FULLY PROTECTED NUCLEOTIDE

Ammonium 5'-O-dimethoxytrityl-adenosine 2'-phosphate

This was prepared by the modified method of Lohrmann, et al. (1966) except that the chilled mixture was brought down to -40° . Work up was performed as described in the literature. The residual concentrated pyridine solution was added dropwise to a large excess of ether with stirring. The white precipitate was collected by centrifugation, washed with ether-cyclohexane, and dried over phosphorus-pentoxide under vacuum. Yield of the product was greater than 60%. The following tests were performed on the sample:

(a) To a solution of an aliquot (30 O.D.) in pyridine (5 ml) was added water (0.10 ml) and then DCC (50 mg). After 5 hours at room temperature, chromatography in solvent A showed only a single spot ($R_f = 0.83$) travelling faster than the starting material ($R_f = 0.50$). The product corresponded presumably to 5'-O-dimethoxytrityl-adenosine-2',3'-cyclic phosphate.

(b) Treatment of an aliquot with 80% acetic acid at room temperature for 20 min., followed by paper chromatography, in solvent E, showed the presence of only a single nucleotidic spot, which corresponded to 2'AMP.

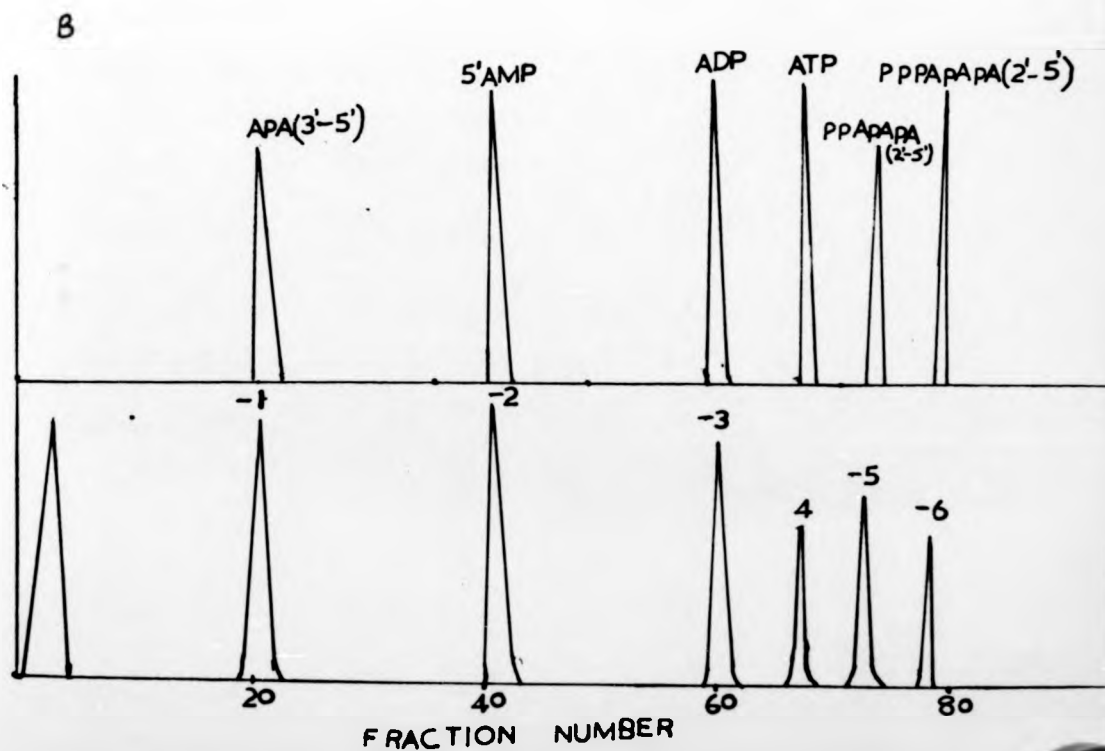
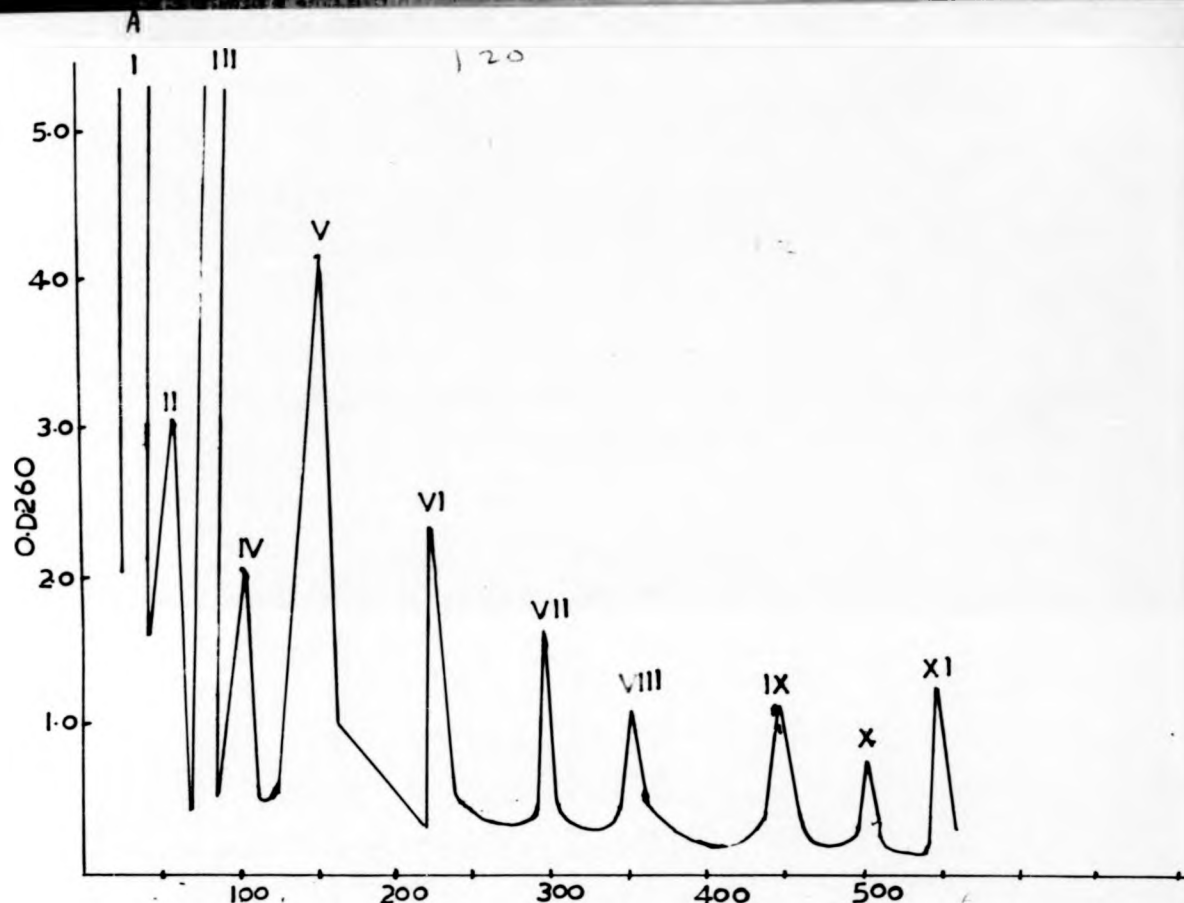
Pyridinium 5'-O-dimethoxytrityl-N⁶,O^{3'}-dibenzoyl-adenosine 2'-phosphate

This was synthesised by the modified procedure of Lohrmann, et al. (1966). An anhydrous mixture of ammonium 5'-O-dimethoxytrityl-adenosine 2'-phosphate (1 mmole) and tetraethylammonium benzoate

FIGURE 1.19 (A) Chromatography of polymeric mixture containing oligonucleotide straight after the work up (0.1 μ mole) on DEAE-Sephadex A-25. Details in text. The distribution of nucleotide material subsequently identified (see Section 1.8.6) in different peaks is given in table below.

<u>Peaks</u>	<u>Fractions Pooled</u>	<u>Identification remarks</u>
I	15-25	Non-nucleotide materials
II	26-53	Non-nucleotide materials
III	65-75	A-3',5'-cyclic phosphate
IV	99-102	A-2'-phosphate
V	104-140	Cyclo-adenylyl(3'-5')-adenylyl (3'-5')-adenylyl(3'-5')
VI	240-250	Unidentified
VII	275-300	ApApA (2'-5')
VIII	350-400	Cyclic trinucleotide
IX	410-450	ApApApA (2'-5')
X	525-550	Pentanucleotide (2'-5')
XI		Unidentified

(B) Determination of charges of synthetic, natural, and commercial products using DEAE-Sephacel previously equilibrated with 100 mM NaCl in 20 mM tris-HCl (pH 7.6/7 M urea). Elutions with a gradient of 100-350 mM NaCl (150 ml/150 ml) in tris-HCl-urea buffer.



(10 mmoles) was treated with benzoic anhydride (20 mmoles) and the mixture was kept for an additional 15 hours in the dark. The reaction mixture was subsequently poured into ice, and the mixture was extracted twice with pentane and then with chloroform (twice). The chloroform extract was dried over sodium sulphate and evaporated in vacuo in the presence of added pyridine. The residual concentrated pyridine solution was added dropwise to an excess of ether-pentane (1:1 v/v), with vigorous stirring. The resulting white precipitate was collected by centrifugation and dried in vacuo over phosphorus pentoxide. The dry powder was now treated with acetic anhydride (20 ml) in anhydrous pyridine (30 ml) for 15 hours. A mixture of anhydrous methanol (20 ml) and pyridine (25 ml) was then added with cooling. After 20 min., the mixture was partly evaporated in vacuo to remove methyl acetate. A mixture (50 ml) of pyridine, ethanol, water (7:1:2 v/v) was then added and the solution was passed through a pyridinium Dowex-50X8 ion exchange resin. The effluent and washings were kept for 2 hours and were then evaporated under reduced pressure with frequent addition of pyridine. The compound was isolated by precipitating it with ether-pentane and dried in vacuo over phosphorus-pentoxide. Yield was 55%. When aliquots of this material were treated with 80% acetic acid (15 mins.) followed by 9N ammonium hydroxide at room temperature for 20 hours, paper chromatography (10 O.D. at 260 nm) in solvent E showed a single spot corresponding to adenosine 2'-phosphate. λ_{max} (water, deprotected product) 260 nm, λ_{min} 232 nm, lit. λ_{max} 259 nm, λ_{min} 232 nm.

Pyridinium 5'-O-monomethoxytrityl,N,O^{3'}-dibenzoyl adenosine 2'-phosphate

The procedure used for synthesis of 5'-O-monomethoxytrityl adenosine 2'-phosphate and for benzylation of the resulting 5'-O-monomethoxytrityl derivative, was very similar to that described previously for the preparation of N,O^{2'},O^{5'},-tribenzoyl adenosine 2'-phosphate from adenosine 2'-phosphate. The yield of the required product was 52%. λ_{max} = 281 nm and 232, Lit. (Lapidot and Khorana, 1963) λ_{max} 281 nm and 235.

1.8.5 SYNTHESIS OF PROTECTED DINUCLEOSIDE PHOSPHATE - PREPARATIVE AND ANALYTICAL STUDIES

(i) Preparative synthesis of dinucleoside phosphate using TPS-C1

To a mixture of the protected mononucleotide (0.5 mmole) the protected nucleoside (2-3 equivalent with respect to the nucleotide), was added in a dry box, and also TPS-C1 (about 2-5 equivalent relative to the mononucleotide). The mixture was kept for 6-8 hours, at room temperature and was then treated with 50% aqueous pyridine (5 ml) with cooling. After 45 min., at room temperature, the solvent was evaporated and the trityl protecting group was removed as follows. The filtered residue was dissolved in 80% acetic acid (30 ml) and the acidic solution was kept for 15 min (for removal of the dimethoxytrityl group), or 3 hours (for removal of the monomethoxytrityl group), at room temperature. The solvent was removed under vacuum and the residue was taken up in chloroform (10 ml) and added to a TEAE-cellulose column (acetate form 2 x 45cm) previously equilibrated with 95% ethanol. Elution was carried out using a linear gradient of triethylammonium acetate; the mixing vessel contained 95% ethanol (2 l) and the reservoir contained a mixture of triethylamine and acetic acid

(0.2 M) in 95% ethanol (2 l), the flow-rate being 2 ml/min. The centre fractions of the main peak were pooled and evaporated with added pyridine. The resulting anhydrous solution was added dropwise to an excess of ether-pentane mixture. The precipitate was collected by centrifugation, washed and dried in vacuo over phosphorus pentoxide. For conversion to the pyridinium salt, it was dissolved in aqueous pyridine and passed through a small column of pyridinium Dowex-50X2 ion exchange resin, and the effluent and washings were lyophilised. The white fluffy powder was stored in a desiccator over magnesium perchlorate. The yield of the protected dinucleoside phosphate prepared and isolated after column chromatography was 35%.

N.B. For preparative purposes, commercial isopropylidene adenosine was used as the protected nucleoside moiety (see Section 1.8.9 for studies on acid hydrolysis of IPA).

(ii) Relative Rate of Synthesis of Protected Dinucleoside Phosphate using:

BS-, TPS-, MS-, Chlorides, Tetrazoles and Imidazoles

An anhydrous pyridine solution (27 ml) of protected nucleotide (9 mmole) and protected nucleoside (10 mmole) was prepared by repeated evaporation of the pyridine. The solution was divided into nine portions (3 ml), each was then treated with the different coupling agent (2 mmole). Aliquots (0.1 ml) were removed at intervals and treated with triethylammonium bicarbonate buffer pH 8.0 (0.1 M, 0.1 ml). The purification of the resulting protected dinucleoside phosphate and removal of trityl groups was as described in the

foregoing experiments, removal of the other remaining protecting groups are discussed below for synthesis of trinucleoside diphosphate.

(iii) Relative Rate of Synthesis of Protected Dinucleoside Phosphate using DCC

A mixture of the protected mononucleotide (1 mmole), the protected nucleoside (2 equivalent with respect to the nucleotide) and dry pyridinium Dowex-50 ion exchange resin (3 g) was rendered anhydrous by repeated evaporation of added pyridine. Anhydrous pyridine (3.0 ml) and DCC (about 2 equivalent with respect to the nucleotides) were added to the residue and the resulting mixture was kept in the dry box with exclusion of light, for 6 days. Aliquots (0.1 ml) were removed at intervals and treated with triethylammonium bicarbonate (0.1 M, 0.1 ml) buffer pH 8.0.

(a) For isolation of the protected dinucleoside phosphate aqueous pyridine (2 ml) was added and the excess DCC was extracted with pentane. The aqueous pyridine solution was kept for 4 hours, at room temperature, the ion exchange resin and the insoluble dicyclohexylurea were removed by filtration. Subsequent removal of the protecting group and isolation of product as discussed above, yielded the dinucleoside phosphate.

(b) For estimation of the relative rate of synthesis of dinucleoside phosphate using individual sulphonyl derivatives listed above, following the addition of triethylammonium bicarbonate buffer pH 8.0 (0.1 M, 0.1 ml) toluene was added after 1 hour, and the solution was evaporated to dryness in vacuo. The residue was dissolved in chloroform (0.1 ml) and applied to silica gel t.l.c., and developed with chloroform:methanol (10:1). The band corresponding to the

desired protected nucleotide (R_f 0.89 with respect to the protected mononucleoside) was scraped off and eluted with chloroform:methanol (5:1). Yield of the protected dinucleoside phosphate material was estimated spectrophotometrically.

1.8.6 SYNTHESIS OF TRINUCLEOSIDE DIPHOSPHATE -
GENERAL PROCEDURE

To an anhydrous pyridine solution (0.5 ml) containing the protected dinucleoside phosphate (10 μ mole) the fully benzoylated mononucleotide (3 equivalent with respect to the dinucleoside phosphate) was added in the dry box, and also TPS-C1 (2.5 equivalent with respect to the mononucleotide), and the sealed reaction mixture was kept for 12 hours at room temperature. An equal volume of water was added with cooling and the mixture kept at room temperature for 18 hours. After evaporating to dryness, the residue was treated, in a well stoppered flask, with 15 M methanolic ammonia (15 ml) for 20 hours. After removal of the solvent, one-fifth of the total products was placed on a DEAE-cellulose (HCO_3^- form) column (2 x 37 cm) which was washed first with water (500 ml) and then eluted with a linear gradient of triethylammonium bicarbonate (pH 7.5). The reservoir contained 3 l of 0.4 M triethylammonium bicarbonate and the mixing vessel an equal volume of water. The various peaks were pooled and assayed for nucleotide and oligonucleotide. The yield of the trinucleoside diphosphate was in the range of 30% from the dinucleoside, and the spectral characteristics were in all ways identical to the monophosphate. The trinucleoside diphosphate was more fluffy compared

to the powdery nature of the monophosphate.

For some applications the sodium salt of the dinucleoside monophosphate and trinucleotide diphosphate was needed. This was prepared as follows. A quantity of nucleotide was dissolved in a small volume of distilled water and applied to a column of SP Sephadex C25 (Na^+ form, 20 x 1 cm). The fractions containing nucleotide were pooled and lyophilised to give fluffy white core.

To obtain oligonucleotide sufficiently pure for biological studies, it was found necessary to subject the triethylammonium bicarbonate product obtained above, to further purification procedures (Astier *et al.* 1976). This method was also useful in the determination of base composition and chain length of the synthetic oligonucleotide when co-chromatographed with authentic biologically isolated material, this will be discussed in the results section. For preparative purposes, a synthetic oligonucleotide sample was subjected to Dowex-1X4, 40 mesh (Cl^- form, 0.2 x 100 cm) ion exchange column. The column was eluted with a linear gradient of 0-0.6 M triethylammonium bicarbonate (pH 8.0) containing 40% ethanol, at flow rate of 22 ml/hour. The separation produced is shown in Fig. 1.19. For analytical purposes (i.e. characterisation of the synthetic products), a mixture of synthetic oligonucleotide (~5 O.D.) biological product (0.2 O.D.) were processed as described above. The synthetic and natural product were co-chromatographed, and the total O.D. obtained was in agreement with the expected O.D. from the summation of the synthetic and natural materials. All products of enzymic treatment in Section 1.8 (i-0) were subjected to this analytical method, in addition to other methods of identification.

1.8.7 SYNTHESIS AND USES OF ARENESULPHONYL
CONDENSING AGENTS

Synthesis of arenesulphonyl triazoles, tetrazoles
and imidazoles

The arenesulphonyl triazoles, tetrazoles and imidazoles were synthesised according to Narang, *et al.* (1974), Stawinski, *et al.* (1976) and Yu Berlin, *et al.* (1973) respectively. Spectral characteristics and other properties agreed with the published data.

Rate of sulphonation of isopropylidene adenosine by
the arenesulphonyl chlorides, triazoles, tetrazoles,
imidazoles and DCC

An anhydrous pyridine solution (2 ml) of isopropylidene adenosine (IPA, 30 mg) was treated with each coupling agent (5 mmole equivalent in each case). Aliquots were taken out at intervals and evaporated *in vacuo* with the addition of toluene to remove pyridine completely. The residue examined by silica gel t.l.c. was developed in chloroform:methanol. U.V. absorbing bands observed corresponded to isopropylidene adenosine and its sulphonated product. The mobilities of isopropylidene adenosine and its 5'-O-sulphonated derivatives are as follows:

<u>Compound</u>	<u>R_f</u>
Isopropylidene adenosine	1.2
Sulphonate derivatives of isopropylidene adenosine derived from:	
BS-Cl	1.4
BS-imidazole	1.5
TPS-Cl	1.7
TPS-imidazole	1.85
TPS-triazole	1.9
TPS-tetrazole	1.95

These compounds were isolated with chloroform:methanol (5:1). Yields were estimated spectrophotometrically (ϵ 14,000).

1.8.8 SYNTHESIS OF OLIGONUCLEOTIDE TRIPHOSPHATE

The oligonucleotide monophosphate (see wheat shoot phosphorylation) (0.1 mmole) was converted into its pyridinium salt with the Dowex-50 X-8 (pyridinium form) cation exchange resin. The tributylammonium salt was prepared by the addition of tributylamine (2 equivalents) and the product was dried by the addition and evaporation of pyridine and DMF. To a solution of the anhydrous tributylammonium salt in DMF (1 ml) was added carbonylbisimidazole (80 mg, 0.5 mmol). The reaction was monitored by cellulose t.l.c. (solvent F). After the completion of the reaction, methanol (50 μ l) was added, and the solution was kept for 10 min. Tributylammonium pyrophosphate (0.5 mmol) prepared from pyridinium salt by addition of 4 equivalents of tributylamine in DMF (5 ml) was then added dropwise with stirring. The mixture was kept for several hours at room temperature and then evaporated to dryness. The 2-5A was isolated by chromatography on columns of DEAE-Sephadex A-25 (HCO_3^- form 0.4 x 100 cm) with a linear gradient of triethylammonium bicarbonate buffer pH 8.0 (0.05 M-0.6 M). The triphosphorylated core was eluted with buffer concentration of 0.4-0.55 M. The products were repurified by the method of Asteriadias, et al. (1976).

1.8.9 KINETIC STUDIES OF ACID HYDROLYSIS OF 5'-NUCLEOSIDE AND NUCLEOTIDE DERIVATIVES

Determination of rates of acid-catalysed hydrolysis of 5'-adenosine derivatives, namely,

- (i) 5'-O-trityl-
- (ii) 5'-O-MMT-
- (iii) 5'-O-MPDA-
- (iv) 5'-O-pixyl-

5'-O-derivatives of adenosine

Samples of the 5'-O-derivatives of adenosine (0.005 g) were dissolved in acetic acid (80%, 1 ml) at 22°. After suitable intervals of time, aliquots (0.1 ml) were removed and treated with ammonia (0.88, 0.1 ml). The basified product was lyophilised and applied to a Whatman No. 42 paper chromatogram, which was then developed in system A (ascending). For each hydrolysis time, equal areas of chromatogram containing the spots corresponding to adenosine and unchanged 5'-O-derivatives of adenosine, and a blank were cut out and allowed to soak in 0.1 N HCl (5 ml) at 20° for 24 hours. The optical densities (O.D.s) of both eluates were measured against the blank at λ_{max} 260 nm.

Straight lines were obtained by plotting

$$\log_{10} \left[\frac{\text{O.D. adenosine} + \text{O.D. 5'-o-x-derivative}}{\text{O.D. adenosine}} \right]$$

against time. The half-times ($t_{1/2}$) of hydrolysis of the various derivatives are shown in Fig. 1.20.

Determination of rate of acid-catalysed hydrolysis of 5'-O-derivatives of 2'-AMP

The ammonium salt of 5'-O-derivatives of adenosine 2'-phosphate (0.1 g) was dissolved in acetic acid (80%, 1 ml) at 22°. After intervals of time, aliquots (0.1 ml) were treated as above. The products were analysed in a Whatman No. 42 paper chromatogram,

which was developed in solvent B. The protected and unprotected adenosine 2'-phosphates were eluted and estimated spectrophotometrically.

After suitable intervals of time, samples (0.2 ml) were removed from a substrate solution (0.01 g), in acetic acid (80%, 1 ml), and added to ammonia (0.88, 0.2 ml). These were analysed as above, and the results plotted in the same way.

Determination of rate of acid-catalysed hydrolysis of isopropylidene adenosine

Isopropylidene adenosine: Commercial isopropylidene adenosine (0.005 g) was treated as described above, and the results are plotted. The half-time of hydrolysis is shown in Fig. 1.20.

Kinetics of hydrolytic cleavage of ApA(2'-5') to adenosine and 2'(3')-phosphates

(i) Acid-catalysed isomerisation of ApA(2'-5')

Ammonium salt of ApA(2'-5') (10 O.D. units at 260 nm, ϵ , 28,000) was dissolved in acetic acid (80%, 1 ml) at 25°. After suitable intervals of time, aliquots (0.2 ml) were treated with 0.88 M aqueous ammonia (0.1 ml) and then lyophilised. The residue was dissolved in *tris*-hydrochloride buffer (pH 7.5, 0.1 ml, 0.005 M) containing pancreatic ribonuclease (50 μ g) and the solution was maintained at 37°. After 12 hours, the products were lyophilised and applied to a strip of Whatman No. 42 paper, and developed with solvent E. The undegraded dinucleoside phosphate was estimated by direct comparison with known amounts of A2'p5'A. After 100 mins., it was found that the undegraded A2'p5'A > 99%.

(ii) Acid-catalysed isomerisation of adenylyl(2'-5')isopropylidene adenosine

This was performed as described under (i) above. The undegraded

dinucleoside phosphate was estimated to be greater than 99%.

(iii) Acid-catalysed degradation of adenylyl(2'-5')adenosine

The ammonium salt of the protected A2'p5'A was treated as above (in the isomerisation experiment) with acetic acid (80%, 1 ml). The neutralised, lyophilised spots were applied directly to a strip of Whatman No. 42 paper, and developed with solvent E. Very small quantities of products of hydrolysis (adenosine, and adenosine 2'(3') phosphates) were estimated by direct comparison. Approximately 0.2% degradation was observed after 100 hours.

(iv) The above experiment was repeated using adenylyl(2'-5')isopropylidene adenosine. Approximately, 0.4% degradation was observed after 100 hours. Products of degradation were analysed by direct comparison with known amounts of adenylyl (2'-5')isopropylidene adenosine. Because of the low level of degradation and isomerisation, it was not found necessary to study the kinetics of hydrolysis and isomerisation.

1.9 RESULTS AND DISCUSSION

1.9.1 SYNTHESIS AND RATE OF ACID HYDROLYSIS OF 5'-PROTECTED ADENOSINE DERIVATIVES

One of the difficulties in oligonucleotide synthesis is the problem associated with the preparation of protected derivatives. The synthetic methods discussed in the early literature were very difficult to follow and apply in terms of reproducibility of yield and isolation. Studies were most interested in obtaining derivatives for subsequent coupling

to form oligonucleotides. More recently, other protecting groups have been introduced to increase the yield of products.

The approach used by Lohrmann et al. (1966) was adopted for the synthesis of the 2'-5' linked oligoadenylate as this had given satisfactory results for the 3'-5' linked derivatives.

The method of Schaller et al. (1963) for MMT adenosine which allegedly gave good yields, did not give appreciable amounts of product and the approach was abandoned as the MMT group was not sufficiently acid-labile.

The trityl group was also not used because of its high stability in acid and also difficulties in obtaining good yields even after various modifications were introduced for the synthesis, in order to increase the yields.

Pixyl adenosine was synthesised in good yield, but it was too labile in some chromatographic systems (e.g. silicic acid). This prevents its use in our synthesis of 2-5A oligonucleotide.

We introduced a novel 5'-protecting group, namely MPDA. Its synthesis in pyridine/DMF gave the highest yield (O.D. units). The difficulty encountered here involves the failure to obtain crystalline adenosine derivatives of MPDA. This is similar to the observation of other workers while trying to obtain crystals of 5'-protected adenosine derivatives.

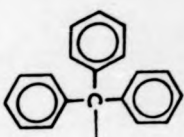
The dimethoxytrityl group was consequently used for the protection of the 5'-hydroxyl group in the majority of the syntheses, Table 2.

It was of interest for the synthesis of 2-5A to compare the relative lability towards acid of a number of protecting groups. The conditions chosen were 80% acetic acid at room temperature.

FIGURE 1.20 Rates of acid hydrolysis of protected
adenosine derivatives in 80% acetic acid.

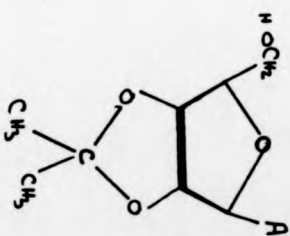
RATES OF HYDROLYSIS OF PROTECTED ADENOSINES IN 80% CH₃COOH AT 22°

5'-DERIVATIVES

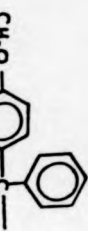


$$t_{1/2} = 23 \text{ h}$$

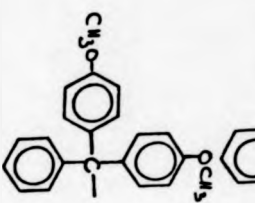
2',3'-DERIVATIVE



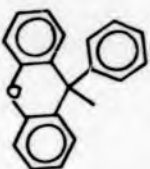
$$t_{1/2} = 19.7 \text{ h}$$



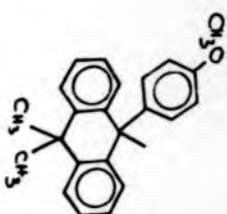
$$t_{1/2} = 1 \text{ h}$$



$$t_{1/2} = 15 \text{ min}$$



$$t_{1/2} = 8.5 \text{ min}$$



$$t_{1/2} = 45 \text{ min}$$

Fig. 1.20

TABLE 2

CONDITIONS FOR SYNTHESIS OF ADENOSINE DERIVATIVES

Compound	Time of Reaction	Temp. of Reaction °C	Solvents as Media	Extraction and Isolation of Products	Yield %
5'-O-MMT-ado	3 days	25	DMF	Ethyl acetate and cyclohexene	30
5'-O-MMT-ado	3 days	35	DMF/pyridine	Ethyl acetate and cyclohexene	20
5'-O-MMT-ado	3 days	25	Pyridine	Ethyl acetate and cyclohexene	10
5'-O-DMT-ado	5 hours	25	DMF	Ethyl acetate, ether-pentane	30
5'-O-DMT-ado	5 hours	40	Pyridine	Ethyl acetate, ether-pentane	20
5'-O-DMT-ado	5 hours	25	Pyridine	Ethyl acetate, ether-pentane	15
5'-O-DMT,N,N,O,O,tetra-benzoylado	3 hours (BzCN)	25	DMF	Ethyl acetate, ether-pentane	28
5'-O-DMT,N,N,O,O,tetra-benzoylado	3 hours (BzCN)	25	Pyridine	Ethyl acetate, ether-pentane	20
5'-O-DMT,N,N,O,O,tetra-benzoylado	3 days (BzCl)	25	Pyridine	Ethyl acetate, ether-pentane	11

From the table, it can be seen that DMT was the most favourable group for our purposes having a half-life of 15 minutes (Table 2 & Fig. 1.20).

In an attempt to obtain a protecting group which gave crystalline stable derivatives, the MPDA group was investigated. Some of its characteristics are shown in Tables 3. In order that our intermediate protected dinucleoside phosphate may be extended from its 5'-OH end, it is necessary that its terminal 2',3'-diol system should, like the 5'-OH group be protected with an acid-labile function, while the 2'-OH group vicinal to the internucleotidic linkage, be protected by base labile group. For the latter condition the benzoyl group was chosen for our synthesis of 2-5A, while the isopropylidene group was chosen for the protection of the 2',3'-diol system, after the acid lability of the isopropylidene derivative was studied (Fig. 1.20). It can be seen that under similar conditions, the isopropylidene adenosine undergoes acidic hydrolysis at a much slower rate than the corresponding 5'-O-DMT adenosine, and that during the time required for the complete removal of the DMT group ($t_{1/2}$ 15 min.), no detectable acidic hydrolysis of the isopropylidene ($t_{1/2}$ 19.7 hours) group took place. This encouraged the use of the latter group, whose half-life in acetic acid (80%) is 19.7 hours, the group was however completely removed with formic acid (90%) in 1.5 hours. It was also found that the conditions required for the complete removal of the isopropylidene and DMT groups from their adenosine derivatives in formic acid (90%) and acetic acid (80%) respectively, are so mild (ca. 1.5 hours and 1/4 hours respectively), that the extent of concomitant phosphoryl migration is immeasurably small. In order

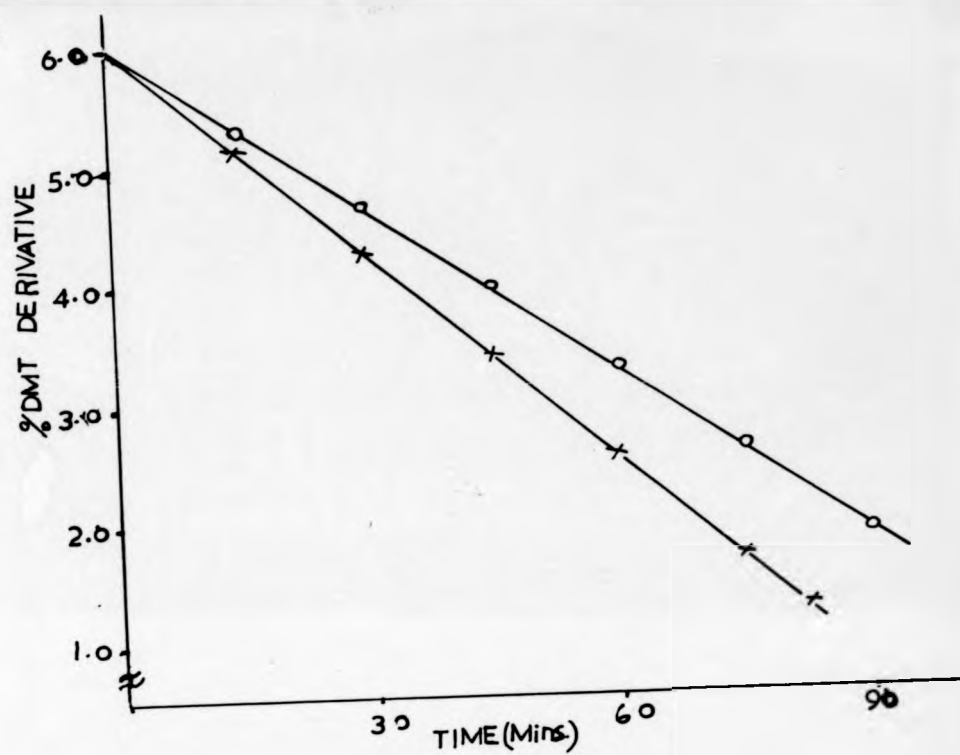
to obtain an estimate of the latter, aliquots of partially protected dinucleoside phosphate were allowed to react with formic acid (90%) and acetic acid (80%) for 3 hours, and 0.5 hours respectively at 25⁰, and the neutralised products then treated with pancreatic ribonuclease. Examination of the resulting digest by paper chromatography revealed that > 99% of the dinucleoside phosphate was undegraded. It follows that the migration of the internucleotidic linkage, to give pancreatic ribonuclease-digested ApA(3'-5') (Brown and Todd, 1953) did not occur in these time intervals. Therefore, no phosphoryl migration would be expected to occur in the time required for the hydrolysis of either the isopropylidene or DMT groups.

When oligonucleotides were treated with dilute acid, hydrolytic cleavage to nucleotide and nucleoside 2'(3')-phosphates was observed in addition to phosphoryl migration (Brown et al., 1956). In order to be certain that such migration will not occur under the acidic conditions used here, acidic hydrolysis of the dinucleoside phosphate in formic acid (90%) and acetic acid (80%) was investigated. It was observed that under these conditions the only product obtained following acid treatment is the unhydrolysed dinucleoside phosphate.

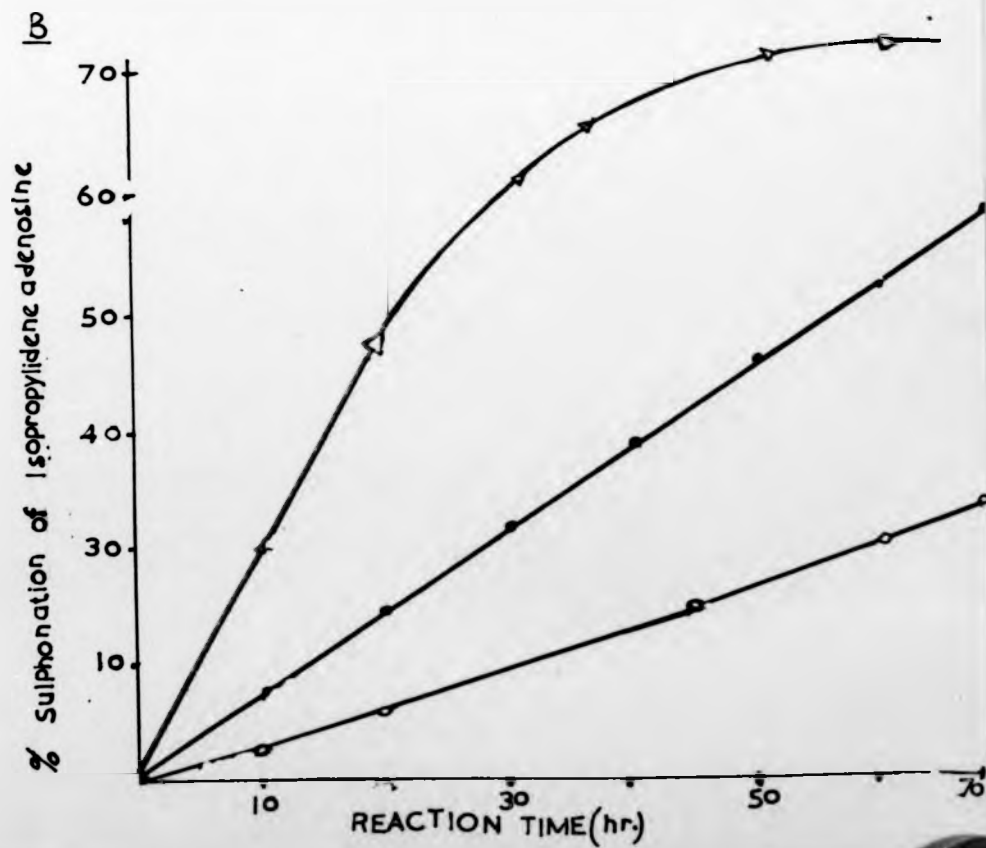
The acid-stability of the 2-5A is probably not surprising, because Smith, et al. (1962) while using acetic acid (80%) observed that during the removal of acid-labile 5'-protecting group functions, from the dinucleoside phosphate (3'-5') after several hours at room temperature, phosphoryl migration occurred to a significant extent. Thus, 2-5' linked oligomer was obtained in a significant amount. This and other studies

- FIGURE 1.2] (A) Hydrolysis of 5'O-DMT derivatives in 80% acetic acid at 24 (o) 5'O-DMT-adenosine, (x) 5'O-DMT dinucleoside phosphate.
- (B) Rates of sulphonation of isopropylidene adenosine by (Δ) TPS-Cl, (●) TPS-imidazole and (o) MS-tetrazole and BS-tetrazole.

A



B



(Smrt and Sorm 1962) indicated that the 2'-5' linked oligomer is more acid-stable than the 3'-5' linked oligomer.

On the assumption that the rate of hydrolysis of the DMT group of the partially protected dinucleoside phosphate derivative might differ from the 5'-O-DMT adenosine equal quantities of the latter and of the ammonium salt of protected dinucleoside phosphate were dissolved together in acetic acid (80%) and allowed to stand at 25°. After suitable intervals of time, aliquots of the reaction solution were removed, neutralised with aqueous ammonia and separated by paper chromatography, into starting materials and products, which were estimated spectrophotometrically. Fig. 1.21A is a plot of the results. Half-time of hydrolysis of the 5'-O-DMT group from protected dinucleoside phosphate (10 mins.) is approximately 5/8ths that of the nucleoside derivative (15 mins.). The difference is possibly due to the participation of the internucleotide phosphodiester function in the hydrolysis of the former.

In devising a new 5'-O-protecting group, we retained the bulky polycyclic ring structure similar to the trityl group, which ensures the same regioselectivity property of the new protecting group. We therefore embarked on the synthesis of MPDA chloride as a possible protecting group for 5'-hydroxyl of nucleosides. To avoid the problems of the ready aromatisation of the B ring of the anthrone moiety, it was necessary not to have the hydrogen atom attached to the 9-position. Hence the starting material chosen for our protecting group was 9,9,-dimethyl anthrone prepared by the method of Curtin et al. (1960).

TABLE 3

PAPER CHROMATOGRAPHY OF ADENOSINE DERIVATIVES

Compound	Solvents R _f			
	A	B	C	D
Adenosine-2'-phosphate	0.11	0.17	0.20	0.20
Adenosine-3'-phosphate	0.12	0.18	0.21	0.12
Adenosine	0.33			
5'-trityladenosine	0.69			
5'-MMT-adenosine	0.61			
5'-pixyladenosine	0.58		0.60	
5'- MPDA-adenosine		0.54		
5'-O-DMT-N,O ^{3'} -dibenzoyladenosine			0.90	
2'-phosphate			0.60	
N,O ^{3'} -dibenzoyladenosine 2'-phosphate				
5'-MMT-adenosine 2'-phosphate	0.56			
5'-DMT-adenosine 2'-phosphate	0.53			

A = 2-propanol:concentrated ammonia:water (7:1:2, v/v)

B = Isobutyric acid:ammonia:water (66:1:33, v/v)

C = Ethanol:ammonium acetate (1 M) pH 7.5 (7:3, v/v)

D = Saturated aqueous ammonium sulphate:sodium acetate (1 M):
isopropanol (79:12:2 v/v)

Treatment of this with the Grignard reagent p-methoxyphenylmagnesium bromide gave MPDA alcohol. The corresponding chloride was prepared by the action of thionyl chloride and used without purification to produce the adenosine 5'-MPDA (Fig. 1.8). Because of the difficulty in obtaining crystalline 5'-MPDA-adenosine, it was not possible to use MPDA-Cl during the synthesis of 2-5A.

1.9.2 SYNTHESIS OF PROTECTED NUCLEOTIDE AND OLIGONUCLEOTIDES: GENERAL PROCEDURE

Comparatively few problems were encountered when intricate attention was paid to all the steps involved in the synthesis of protected nucleotide. The route followed for the synthesis of adenylylyl(2'-5')adenylylyl(2'-5')adenosine closely followed that of Lohrmann and coworkers (1966) from 2'AMP using DMT and benzoyl groups for the protection of the 5'- and 3'-hydroxyls respectively. The overall scheme is shown in Fig. 1.7. Benzoylation of the nucleotide using benzoylcyanide proceeded faster and cleaner than when benzoyl chloride was used. The isopropylidene group was chosen for the protection of the 2',3'-diol end, as mentioned earlier, because it had a suitable half-life in acetic acid to allow the removal of the 5'-dimethoxytrityl group during the synthesis. The condensing agents used were TPSimidazole and TPSCl and DCC. It was found that the rate of condensation using DCC was slow, but work-up of the reaction product was generally simpler. TPS & MS condensing agents caused extensive sulphonation of the 5'-hydroxy group (Fig. 1.218) thus blocking possible condensation. In the synthesis of ApA(2'-5'), the rate of

5'-hydroxyl sulphonation increases in the order DCC=BST=TST < MST < MSimidazole < TPSimidazole < TPSCl < MSCl.

While evaluating the usefulness of various condensing agents it was found that until a better phosphorylating agent was found (there is need for one), in the phosphodiester synthesis, the sulphonyl chlorides produce extensive sulphonation of the 5'-hydroxyl group, darkening of the reaction, and relatively low yield. DCC produced less sulphonation, no darkening of reaction media, but is extremely slow and the yield is just average. It is our opinion that the sulphonyl triazolides and tetrazolides are too reactive (especially when amino and phosphate groups are left unprotected), leading to undesirable side products and low yields.

It is our opinion that up to date, the imidazolides are the best compromise in phosphodiester synthesis. Sulphonylimidazole's slower rate of reaction compared to the chloride is more than compensated for by the improved overall yield and by the fact that the products are relatively cleaner when the former is used. The overall yield of the fully protected adenylyl-2'-5'-adenylyl-2'-5'-adenosine was low due to unavoidable losses by side reactions and inefficient steps in a multistage synthesis.

Adenylyl-2'-5'-adenylyl-2'-5'-adenosine was phosphorylated using wheat shoot phosphotransferase (see page 145). The 5'-phosphate obtained was converted into the 5'-triphosphate by the modified method of Hoard and Ott (1965).

The identity of the phosphorylated adenylyl-2'-5'-adenylyl-2'-5'-adenosine was confirmed in the following manner:

(1) Stability to RNase T₂

RNase A

(2) Hydrolysis by alkali under 0.3 M KOH.

(3) The phosphorus to adenine ratio was 5:3.

(4) Hydrolysis by snake venom phosphodiesterase as described in experimental.

Other methods of characterisation of the triphosphorylated core and its other intermediates are discussed in the next section.

1.9.3 CHARACTERISATION OF OLIGONUCLEOTIDES

1.9.3.1 GENERAL ENZYMIC CHARACTERISATION

All the synthetic products were resistant to pancreatic ribonuclease P₁, P₂ and ribonuclease T₁, T₂. This excludes the presence of any significant amount of 3'-5' linkage in the oligonucleotide. In the synthesis of oligonucleotides and polynucleotides with "natural" 3'-5' linkage, up to date, pancreatic ribonucleases have served as the major and initial tools for estimating the extent and presence of 3'-5' linkage (Reference Lohrmann et al. (1962)).

The synthetic oligonucleotides (i.e. A2'p5'A2'p5'A, pA2'p5'A2'p5'A, ppA2'p5'A2'p5'A and 2-5A) were all sensitive to the actions of snake venom phosphodiesterase (SVPD), bacterial alkaline phosphatase (BAP) and alkali hydrolysis. The first enzyme is a member of the class of phosphodiesterases, which degrade phosphate internucleotide bonds. Thus, whereas the phosphodiesterase from Lactobacillus acidophilus will degrade oligonucleotide to release 3'-phosphate, the action of SVPD releases 5'-phosphate. Thus, when the synthetic products were treated with

SVPD, in all cases only 5'-phosphorylated products were released. Bacterial alkaline phosphatase treatment of the triphosphorylated product yielded a product whose R_f differs from the starting material and which chromatographed in all systems used with the trinucleoside diphosphate (both natural and synthetic). This shows the product of BAP treatment to be the core (2'-5' linked) oligonucleotide. Further usefulness of the BAP treatment was obtained when it was followed by alkaline hydrolysis, this resulted in the identification of A2'p,Ap3' and adenosine, all of which chromatographed in the analytical columns with the commercial materials. These products are in accord with the expected structure of ApApA(2'5'-linked). Similar treatment of commercially available ApA(3'5'-linked), yielded only 3'AMP, and adenosine as the major products and treatment of the natural core yielded products which chromatographed identically with the synthetic material. In determining the charge of the core oligonucleotide, and tri-, di- and mono-phosphorylated oligonucleotide, charges of -2, -6, -5, and -4 were respectively obtained, using the technique described by Kerr and Brown (1978) (see Fig. 1.19B) These charges, and the pattern obtained on BAP digestion, exclude di- or polyphosphate or inverted (5'-5') linkages in the synthetic oligonucleotide and since 2'AMP was the starting material, it is reasonable to conclude that the synthetic products obtained are A2'p5'A2'p5'A, pA2'p5'A2'p5'A, ppA2'p5'A2'pA and pppA2'p5'A2'p5'A.

1.9.3.2 ADENINE:PHOSPHATE

Adenine:phosphate ratio determined as shown in the following tables agrees with the results of general enzymatic digestion. For the synthetic 2-5A,

the adenine:phosphate ratios of two determinations are 1.7:1 and 1.6:1 respectively. The method used for phosphorus assay was found to be satisfactory for determination of small amounts of materials.

1.9.3.3 ALKALI HYDROLYSIS

Alkali hydrolysis of the oligonucleotide also produced further evidence of structure of the triphosphorylated product. Alkali treatment of the core yielded only 2'- and 3'-nucleotide and adenosine, and the phosphorylated products yielded no inorganic Pi. In this respect, the triphosphorylated product yielded pppAp which was identified chromatographically using appropriate markers. In addition, when this peak was eluted, it shows adenine phosphate of 1.41 thus identifying the product as pppA2'(3')p.

Hydrolytic action of the alkali on 2-5A is shown below:

$$\text{pppA2'p5'A2'p5A} \rightarrow \text{pppA2'(3')p} + \text{A2'(3')p} + \text{adenosine}$$

$$\text{p}_\gamma\text{p}_\beta\text{p}_\alpha \text{ and } \text{p}_{2'(3')} \text{ were found associated together with a mole of}$$

oligonucleotide, eluting at the same charge point as pppApApA(2'-5'). The latter was ruled out by the oligonucleotide:phosphate ration, while periodate-Schiff tests excluded ATP, it is therefore most likely that the second product is pppAp(2' or 3'). The final product contained a mixture of 2'(3') terminal linkage. In this regard, Tener,

(1968) noticed that the left or 5'-OH end of many RNA molecules carrying a 5'-phosphomonoester group liberates this end as a nucleoside 2'(3')-5'-diphosphate, whereas most of the other residues are liberated as nucleoside 2'(3')-phosphates, and that the nucleoside diphosphates can be separated from other degradation products by virtue of their higher ionic charge per nucleotide. The t.l.c. of the synthetic and natural materials, agrees to a large extent.

1.9.3.4 TWO-STEP PROCEDURE

Employing the 2-step procedure of Laskowski II (1968), for identification of short-chain oligonucleotides, each product was subjected to the hydrolytic functions of three enzymes: (i) alkaline phosphatase, (ii) snake venom phosphodiesterase, (iii) spleen phosphodiesterase.

The oligomer (in four portions) was first dephosphorylated with the first enzyme according to the procedure described earlier. The dephosphorylated product was identified by t.l.c. and analytical paper chromatography. Inorganic phosphate was assayed as described before, the other duplicate portions of the dephosphorylated products were then divided into two portions each (labelled A^1 , A^2 , B^1 , B^2 , while A^1 , A^2 were treated with snake venom phosphodiesterase as described under experimental. B^1 , B^2 , were treated with spleen phosphodiesterase according to the method of Brownlee (1972). The products were analysed in the usual way. While SVPD produced 5'-nucleotides and nucleosides only, spleen phosphodiesterase did not degrade the core.

In all cases where t.l.c. resolution of products of digestion

was difficult, two dimensional paper chromatography and "finger printing" gave better resolutions.

One of the most satisfactory confirmations of the structure of the synthetic oligonucleotide was obtained from sequential degradation using periodate oxidation, and β -elimination. When these were coupled with enzymic degradation, conclusive evidence for the structure of 2-5A was provided (following table).

1.9.3.5 PERIODATE OXIDATION AND β -ELIMINATION

Periodate oxidation followed by β -elimination, using the method of Schmidt (1968) which employs buffered cyclohexylamine gave satisfactory results; the product was identified by its reduced mobility in PEI-cellulose in 0.75 M potassium phosphate, pH 3.4, by nucleoside to phosphate ratio now changed from 3/5 to 2/5, and by being negative to periodate-Schiff reagent. Aliquots of the product were treated with BAP to yield A2'p5'A, and inorganic phosphate (4 moles Pi/mole nucleoside). The dinucleoside monophosphate was isolated and identified by;

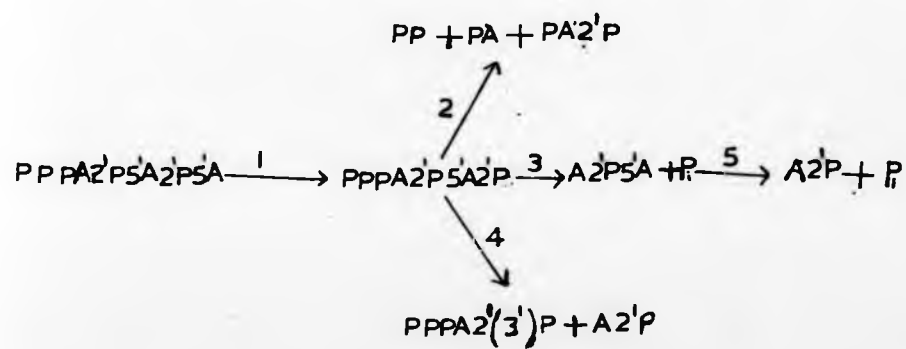
- (a) its increased mobility in PEI cellulose,
- (b) being positive to periodate-Schiff tests,
- (c) being resistant to nuclease digestion,
- (d) nucleoside:phosphate ratio 2:1 (approximately),
- (e) co-migration with natural ApA(3-5) on all chromatographic systems used,
- (f) charge density value -1 on Sephacel eluting with buffered 7 M urea,
- (g) insensitivity to BAP and 5'-nucleotidase,

FIGURE 1.22 Schematic representation of analysis of 2-5A.

- 1 β -elimination
- 2 SVPD digestion
- 3 Bacterial alkaline phosphatase digestion
- 4 0.3 M KOH
- 5 β -elimination

2-5A.

estion



- (h) portion of the material was subjected to a second round of β -elimination and periodate oxidation, and inorganic phosphate and 2'AMP were the only products identified.

Taken together, these results indicate the following:

- (1) That the 5'-terminal linkage is 2'-5'.
- (2) That the 5'-terminal end may bear a triphosphate.

1.9.3.6 GENERAL DISCUSSION: CHARACTERISATION STUDIES

- (i) The 5'-distal linkage is 2'-5'.
- (ii) The 5'-primary alcohol bears α, β, γ -phosphate groups which are released as compound pppA2'(3')p on alkali treatment, this is in agreement with the known action of alkali. In addition, a (3')2'-phosphate group is attached to another nucleoside molecule, and free adenosine was released.
- (iii) Specifically, due to the hydrolytic cleavage of pppApAp by SVPD, it was deduced that the primary hydroxy function bears a triphosphate which is released in form of pyrophosphate (2 μ mole inorganic phosphate/ μ mole of starting material and 5'AMP (Scheme 1.22).
- (iv) Evidence for the nature of the termini is derived from:
 - (a) Sensitivity of the synthetic 2-5A to periodate-Schiff reagent, thus excluding the presence of 2'- or 3'-terminal phosphate

(b) treatment of the synthetic product, 2-5A with SVPD, released 2 μ moles of inorganic pyrophosphate and 5'AMP only. When the products of SVPD digestion were treated with BAP, only inorganic phosphate and adenosine were released.

(c) treatment of the synthetic product with purified BAP alone, released 3 μ mole of inorganic phosphorus/ μ mole of material Heppel, et al. (1962), have shown that alkaline phosphatase hydrolyses pyrophosphates such as ATP).

(d) From the core trimer to pApApA, ppApApA, and pppApApA, a decrease in nucleoside/phosphate in the order 3/2, 3/3, 3/4 and 3/5 occurred.

(v) Evidence for the nature of the linkage was derived from:

(a) The fact that the synthetic products are all insensitive to nucleases such as pancreatic, spleen, micrococcal nucleases whereas they are sensitive to SVPD.

(b) Sequential degradation using periodate oxidation and β -elimination, followed by enzymic digestion, as described above confirmed the 2'-5' linkage.

(c) Treatment of the synthetic product with BAP followed by SVPD yielded only 5'AMP and adenosine, these materials totally accounted for 98% of the synthetic material; this precludes the absence of any other modification which may be produced during the chemical synthesis.

The above discussion has been concentrated on the total identification of the triphosphorylated oligonucleotide, and experiments have been performed on the intermediates obtained during the synthesis:

(i) A2'p5'A and its mono-, di-, and tri-phosphorylated products.

(ii) A2'p5'A2'p5'A and its mono-, di-, and tri-phosphorylated products.

The condition for progressing to the next stage during the synthesis rested on the total product of each step. Tables have been prepared to show the characteristics and identity of some of the intermediates obtained during the synthesis of the triphosphorylated oligonucleotide (pppA2'p5'A2'p5'A).

Products of SVPD digestion were analysed by two dimensional chromatography on PEI-cellulose with 1.5 M lithium chloride and 1 M acetic acid as solvents (Cashel et al., 1969). Two-dimensional chromatography with (dimension I) isobutyric acid: 0.5 M NH_4OH (5:3 v/v) and (dimension II) isopropanol: HCl:water (70:15:15v/v).

SVPD plus BAP digested products were identified by cellulose chromatography and developing solvents are (dimension I), amyl alcohol: methyl ethyl ketone:acetonitrile:ethylene acetate:water:formic acid (40:20:15:1:8 v/v) (dimension II), acetonitrile:ethylene acetate:1-butanol: isopropanol:1 M NH_4OH (35:10;5:17:5: v/v) (Table 4 shows some of the chromatographic data of the products.

RESULTS OF CHARACTERISATION OF OLIGONUCLEOTIDES

Conditions and solvents are discussed in text.

Nucleoside:Phosphate Ratio			
ApA	(2'-5')	2.1	1.06
ApApA	(2'-5')	3	2
pApApA	(2'-5')	2.8	3.1
ppApApA	(2'-5')	3.2	3.97
pppApApA	(2'-5')	3.15	4.96

Digestion with SVPD

	Adenosine	5'AMP	Ratio in μ moles	
ApA	5'pA only		1.12	: 1.32
pApApA	pi:5'AMP		1.08	: 3.15
ppApApA	pi:5'AMP		2.12	: 3.30

Two-Step Procedure

First Step			Spleen Phosphatidyl esterase		
BAP			SVPD		
Product	Ratio in μ moles		Product	Ratio in μ moles	Ratio in μ moles
pApApA (2'-5') ApApA ^(2'-5') ++ pi	(1.2:1.1)		A:5'AMP	(1:2.3)	5'AMP:A 2.2:1.3
ppApApA " ApApA ^(2'-5') ++ pi	(1.2:2.1)		"	"	" "
pppApApA " ApApA ^(2'-5') ++ pi	(1.08:3.2)		"	"	2.3:1.4

Pancreatic Ribonuclease

ApA (2'5')	Negative	resistant
pApApA "	"	resistant
ppApApA "	"	resistant
pppApApA "	"	resistant
ApA (3'5')	Positive	hydrolysed to > 96%
(Commercial)		

Micrococcal Nuclease Digestion

ApA (2'-5')	Negative	resistant
ApApA "	"	resistant
pApApA "	"	resistant
ppApApA "	"	resistant
pppApApA "	"	resistant
ApA (3'5')	Positive	hydrolysed to > 96%
(Commercial)		

Periodate α ,8-elimination + SVPD

			Ratio in μ moles
ApA	(2'-5')	ND	
ApApA	(2'-5')	A:pA2'(3')p	1:1.02
pApApA	(2'-5')	ND	
ppApApA	(2'-5')	ND	
pppApApA	(2'-5')	pAp:pA:pi	1.02:1.02:1.1:2.3

1.10 BIOLOGICAL STUDIES

1.10.1 MATERIALS AND METHODS

General

(a) Human cell lines used were:

(i) Human foreskin fibroblasts (HFF)

(ii) An osteosarcoma cell line, MG63, obtained from Dr. A. Billiau, Rega Institute, Leuven, Belgium.

(b) Foetal calf serum was from Sera-Labs., Crawley Down, Sussex, U.K.

(c) Polynucleotides were a produce of PL Biochemicals, Milwaukee, Wisconsin, U.S.A.

(d) 2'-5' Oligoadenylates were prepared as described.

(e) Semliki Forest Virus (SFV) 5 plaque forming units/cell.

(f) Protein synthesis solution: Overlay medium: 2 x 199 and 1.8%

agar - mixed in equal volume, plus 7% foetal calf serum, 0.04% DEAE Dextra

(g) Tritosol.

Spleen Phosphodiesterase

			Ratio in μ moles
ApA	(2'-5')	Ap2':A	1:1
ApApA	(2'-5')	Ap2':A	2.3:1.2
pApApA	(2'-5')	Resistant	
ppApApA	(2'-5')	"	
pppApApA	(2'-5')	"	

Bacterial Alkaline Phosphatase (BAP)

			Ratio in μ moles
ApA	(2'-5')	Resistant	
ApApA	(2'-5')	"	
pApApA	(2'-5')	ApApA(2'-5'):pi	1.2:1.1
ppApApA	(2'-5')	ApApA(2'-5'):pi	1.2:2.3
pppApApA	(2'-5')	ApApA(2'-5'):pi	1.1:3.4

Alkaline Hydrolysis

			Ratio in μ moles
ApA	(2'-5')	Ap2':A	1:1.1
ApApA	(2'-5')	Ap2':Ap3':A	1:1:1.05
pApApA	(2'-5')	Ap2':Ap3':A:pAp 2'(3')	1 : 1 : 1.1:1.02
ppApApA	(2'-5')	Ap2':Ap3':A:ppAp 2'(3')	1 : 1 : 1.2:0.9
pppApApA	(2'-5')	Ap2':Ap3':A:pppAp 2'(3')	1 : 1 : 1:1.3

Periodate α,β -elimination followed by BAP

			Ratio in μ moles
ApA	(2'-5')	ND	
ApApA	(2'-5')	ND	
pApApA	(2'-5')	ApA(2'-5') + \textcircled{A} + pi	1:1.02:2.1
ppApApA	(2'-5')	ApA(2'-5') + \textcircled{A} + pi	1.1:1.12:2.2
pppApApA	(2'-5')	ApA(2'-5') + \textcircled{A} + pi	1.2:1.1:3.2

\textcircled{A} Oxidised products of adenosine resulting from periodate degradation and β -elimination

TABLE 5

PAPER CHROMATOGRAPHY OF OLIGONUCLEOTIDE

		R _f Solvents				
Compound		A	B	C	D	E
Synthetic	ApA (2-5)	ND	ND	0.80		1.38
	ApApA (2-5)					0.82
	pApApA (2-5)			0.52		0.27
	ppApApA (2-5)					0.15
	pppApApA (2-5)					0.05
Natural	ApA (2-5)			0.82		1.40
	ApApA (2-5)					0.80
	pApApA (2-5)			0.60		0.27
	pppApApA (2-5)					0.05
Commercial	Ap2'	ND	0.21	0.8	ND	1.0 (R _f value relative to Ap2')
	Ap3'	ND	0.20	0.8	0.5	0.5
	ApA (3'5')					1.30
	A:3',5'-cyclic P	0.43	ND	0.20		1.90

A = 2-propanol:ammonium hydroxide:water (7:1:2 v/v)

B = ethanol - 1 M ammonium acetate, pH 7.5 (7:3 v/v)

C = isobutyric-acid-concentrated ammonia in water, pH 3.7 (66:1:33 v/v)

D = saturated aqueous ammonium sulphate:1 M NaOAc:isopropyl alcohol
(79:19:12 v/v)

E = 1-propanol:conc. ammonia:water (55:10:35 v/v)

1.10.2 CELL CULTURE

Cells were cultured in the Glasgow modification of minimal essential medium supplemented with non-essential amino acids containing foetal calf serum (10% v/v), Benzoylpenicillin (100 units/ml) and streptomycin (100 µg/ml). Incubation was at 37° in a 5% CO₂/air atmosphere. Determination of DNA concentration with diphenylamine was performed according to the method of Giles and Myers (1965).

1.10.3 INTERFERON INDUCTION AND ASSAY

Interferon Assays

These were performed in the HFF and were based on the method of inhibition of nucleic acid synthesis as described by (Atkins, 1971). The titres obtained are 3.40 and 3.68 log₁₀U/10⁶ cells, for the interferon induction and superinduction respectively. Each titre being an average of duplicate experiments.

Interferon Induction

The basic procedure is outlined below. For simple induction at 37°, confluent monolayers of MG63 cells on 50 mm dishes were washed four times with serum-free medium (2 ml) and then incubated with (I)_n(C)_n solution (50 µg/ml) in serum free medium for 1 hour at 37°C. The supernatant was drawn off, the cell sheets washed several times more with serum-free medium (2 ml) and finally incubated overnight in maintenance medium (i.e. containing 2% foetal calf serum 2 ml). Fluids were collected and stored at -20° until required for assay.

Superinduction

The medium was removed from the cells and they were washed four times with 2.0 ml of serum-free medium. $(I)_n \cdot (C)_n$ (50 μ g/ml) and cycloheximide (50 μ g/ml) were added in solution (2 ml) and incubated at 37°C for 1 hour. $(I)_n \cdot (C)_n$ was removed from the culture, and the cells washed with maintenance medium (2 x 2 ml). Maintenance medium (2 ml) containing cycloheximide (50 μ g/ml) was added and incubated for 5 hours at 37°C. Actinomycin D (5 μ g) was added and incubated for a further $\frac{1}{2}$ hour at 37°C, after which the medium was removed and the cells washed with maintenance medium (4 x 2 ml). Maintenance medium was added and the cells incubated overnight at 37°C. The medium was harvested and dialysed against PBS at 4°C for three days, with buffer changes after three hours and again the next day. The interferon solution was treated with 20 ml of RNAase solution for 1 hour at 37°C prior to assaying.

1.10.4 CELL PERMEABILISATION AND CHARACTERISATION OF PERMEABLE CELLS

Cells to be made permeable were plated about 3×10^6 per 35 mm tissue culture dish at least 18 hours before the experiment began. The plates were each washed four times with PBS and then incubated at 37°C in modified Eagles medium (2 ml) without serum, but containing sodium chloride (4.2 g per 100 ml of medium). The extent of permeability was assessed by uptake of Trypan Blue. It generally took about 75 min. for 90% of the cells to stain, after which the hypertonic medium was washed and the cells were characterised as described in the

under results. The reseal of permeable cells proceeded as follows: as soon as > 90% of cells were stained, the hypertonic medium was aspirated and cells were washed with PBS, and later with complete medium (1 ml). The extent of resealment was tested using Trypan Blue stain.

On microscopic examination, the permeable and intact MG63 cells were very similar, and most of the visible cell components under high power microscope appeared normal in permeable cells.

Assays were performed to determine retention of protein (Lowry, et al., 1951) and nucleic acid materials (Giles and Myers, 1965).

It was found that while the intact cells contained 120 μg protein per 10^5 cells, permeable cells contained at least 100 μg protein per 10^5 cells. Both groups of cells were found to contain roughly equal amounts of nucleic acid materials (about 40 μg each).

1.10.5 PROTEIN SYNTHESIS IN PERMEABLE CELLS

Experiments to determine the extent of protein synthesis in permeable cells employed the method of Castellot II (1978).

1.10.6 REVERSIBILITY OF PERMEABLE CELLS

As a test for reversibility of permeable cells, cells were counted as a function of time after all cells were made permeable and resealed. At various intervals of time

media were withdrawn, and the cells resealed as described below. The growth rates of such permeabilised and then resealed cells were monitored for increase or decrease in cell number. To demonstrate the viability of permeable cells directly, cells were made permeable, and then exposed for 1 hour to the protein synthesis solution, containing (^{35}S)methionine ($0.4 \mu\text{Ci ml}^{-1}$, 400 Ci mol^{-1}). The protein synthesis solution was aspirated and replaced with complete modified Glasgow's medium twice at 15 min. intervals. As a control, intact cells were also exposed to the same ^{35}S -methionine ($0.4 \mu\text{Ci/ml}$, 400 Ci mol^{-1}) containing protein synthesis solution for 1 hour. After the time interval, cells were washed once with PBS and 3 ml of 5% TCA were added for 30 mins. at 4° . The cells were washed five times with 5% TCA, and digested with sodium hydroxide (1.0 ml, 0.1 M) for 30 min. at 37° , and neutralised with hydrochloric acid (0.1 ml, 1.0 M HCl). The radioactivity of the entire sample was measured in 10 ml of Tritosol.

1.10.7 PERMEABILISATION OF MG63 CELLS AND TREATMENT WITH 2-5A

MG63 cells were plated at about 3×10^6 cells per 35 mm tissue culture dish for 24 hours. The cells were made permeable by washing with PBS and then incubated at 37°C in 2.5 ml of Dulbecco's modified Eagle's medium containing 10% calf serum (Flow) at 37°C (in a humidified 5% CO_2 atmosphere) without serum, but containing 4.2% NaCl. Routinely, 2-5A was added with the hypertonic medium to the cells. The preparation of 2-5A used for the experiments described here was purified by the procedures described earlier in this study, Section 1.8.6. When approximately 90% of the cells were permeable to staining by

Trypan Blue, the hypertonic medium was removed, the cells were washed with PBS and 1 ml of complete medium minus methionine and containing the protein synthesis solution. ^{35}S -methionine ($0.8 \mu\text{Ci/ml}$, ($900\text{--}1000 \mu\text{Ci/mmol}^{-1}$) was added to the cells. After 2 hours (or interval of time as appropriate), cells were washed with PBS, and 5% TCA (1 ml) was added for 45 mins. at 4°C . The cells were then washed three times with 5% TCA, digested in 0.1 M NaOH (1.0 ml) for 45 mins. at 37°C and neutralised with 1.0 M HCl (0.1 ml). The entire sample was counted by dilution into tritosol (10 ml). Incorporation of ^{35}S -methionine into permeable cells in the presence of different concentrations of 2-5A is expressed as a percentage of the control (no 2-5A).

1.10.8 INHIBITION OF SEMLIKI VIRUS GROWTH BY 2-5A

Monolayers of HFF cells were grown (as described in section 1.10.2) at 3×10^6 cells/ml dispersed in 35 mm petri dishes in 3 ml aliquots and incubated overnight at 37°C . Fluids were removed from the plates and replaced with 0.5 ml virus preparation diluted to give either 0.2 or 2.0 Pfu/cell to each plate, and the plates were incubated for 1 hour at 37°C , each plate was then permeabilised and treated with 2-5A, exactly 1.5 hours after infection with the virus; the experiments were performed in duplicate for each infection. The plates were incubated for 1 hour at 37°C , after which the medium was removed, and

the tissue culture washed twice with 199 2% NBS (1 ml). The medium 199 + 2% NBS (2 ml) was added to all plates and incubated for 24 hours at 37°C. The supernatant fluids were harvested, pooling duplicates. The samples were assayed for virus yield by plaquing on HFF cells. This was performed as follows:

Fluids were removed from HFF plates and replaced with dilutions obtained from the harvested supernatant fluid. Each dilution was assayed in duplicate.

The HFF plates were incubated at 37°C for 1 hour, after which the fluids were removed and overlaid with overlay medium (4 ml). This was left to set and then incubated for 4 hours at 37°C. The plates were then stained with neutral red for 2 hours, and then plates were counted. The extent to which virus yield is depressed after 12 and 24 hours is shown in table 6.

1.10.9 THE EFFECTS OF 2-5A ON CELL GROWTH

Effects on cell growth were measured by counting viable cells. Replicate cultures (5×10^4 cells per 35 mm culture dish) were permeabilised and treated with each of the various concentrations of 2-5A. After 96 hours later, when control cultures without 2-5A had a 3-5 fold increase in cell number, cells were counted by using 0.1% Trypan Blue for viability determination. The effect of 2-5A on a given cell line was expressed as a percentage of control growth.

1.10.10 RESULTS AND DISCUSSION

1.10.10.1 CHARACTERISTICS OF PERMEABLE CELLS

In modified Eagle's medium, containing 4.2% NaCl, MG63 and HFF cells were made permeable to Trypan Blue, while the gross morphology of the cells as seen under the microscope, was preserved. The time required for > 90% of the cells to stain ranged from 60-75 minutes, test plates were used to monitor for Trypan Blue uptake during the permeability procedure to ensure that maximum permeability of the cells was achieved for each experiment. Microscopic examination under high power showed that the permeable and intact MG63 and HFF cells were very similar. Assay performed to determine the retention of nucleic acid components and protein showed that while the intact cells contained 120 μg protein per 10^5 cells, permeable cells contain 100 μg protein per 10^5 cells and both cells were found to contain equal amounts of nucleic acid materials. Fig. 1.24a shows that permeable MG63 cells incorporated [^{35}S]methionine into acid-insoluble material. While protein synthesis in permeable cells was dependent on exogenously supplied protein synthesising reagents this contrasted with the situation in intact cells. This requirement shows that permeable cells were actually responsible for the ^{35}S -methionine incorporation, and not the < 10% unpermeabilised cells which are present in the permeabilised group. However, a 20% drop of control incorporation of ^{35}S -methionine was observed on permeabilisation.

FIGURE 1.23 A Effects of 2-5A on the kinetics of protein synthesis in permeabilised MG63 cells. Cell monolayers were made permeable as described in the text and incubated in labelling medium in the presence or absence of 2-5A at a final concentration of 100 μ M. At the times indicated, control plates containing cells not permeabilised (o), control plates with permeabilised (\bullet) cells, and plates with 2-5A (Δ) were treated as described in the text.

B Effects of 2-5A on the kinetics of protein synthesis in permeabilised MG63 cells. The plot shows the protein synthesis of cells in presence of 10 μ M (\bullet), 50 μ M (Δ) and in absence (o) of 2-5A.

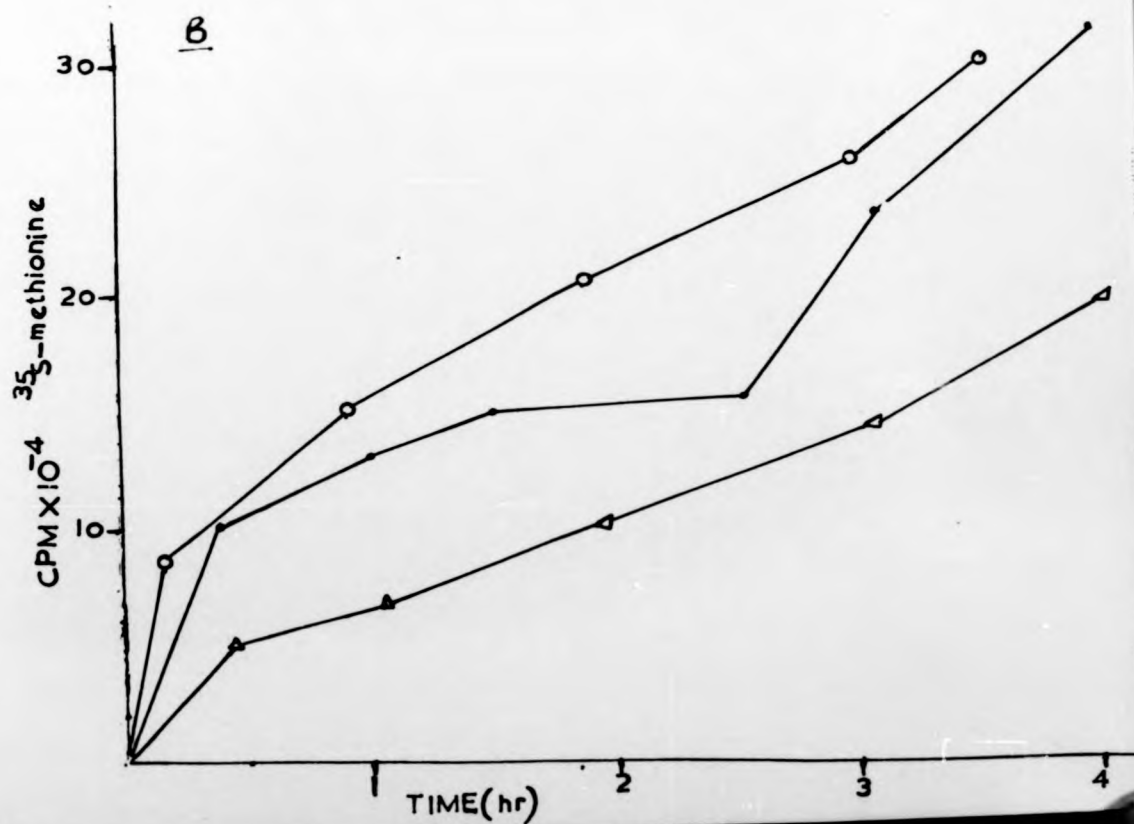
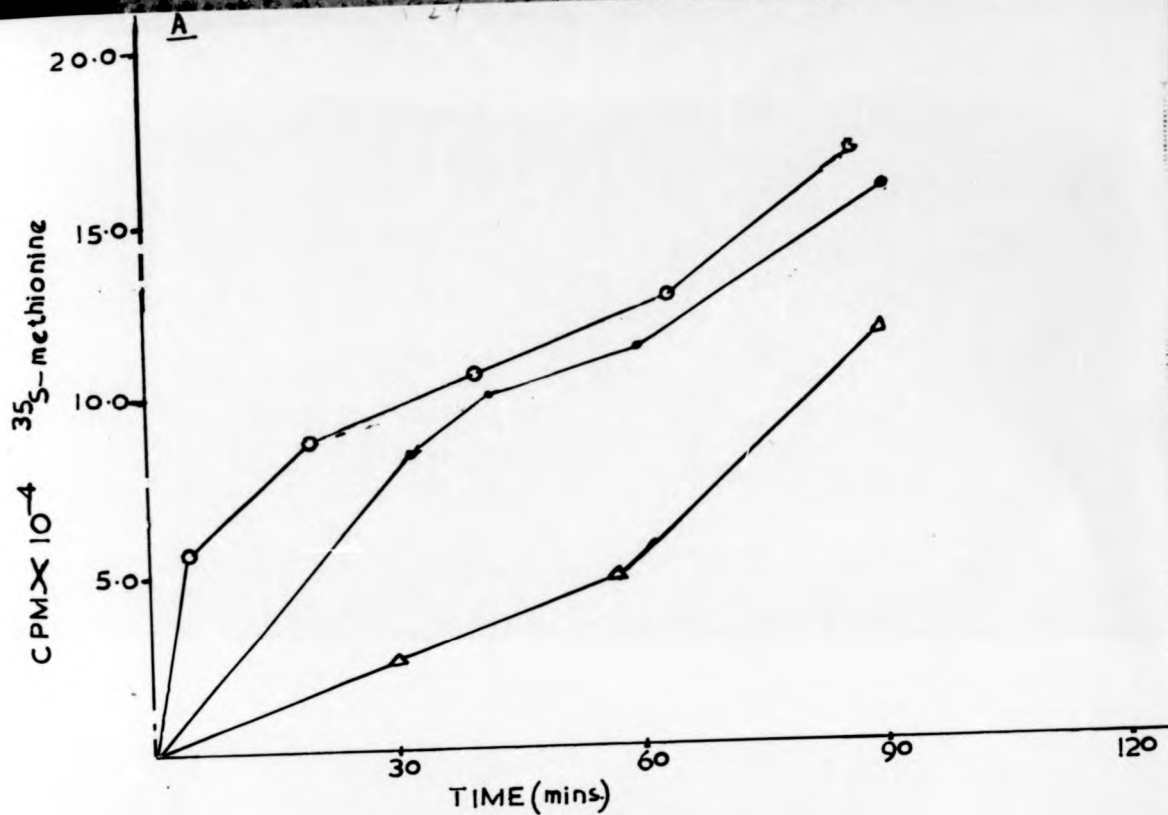
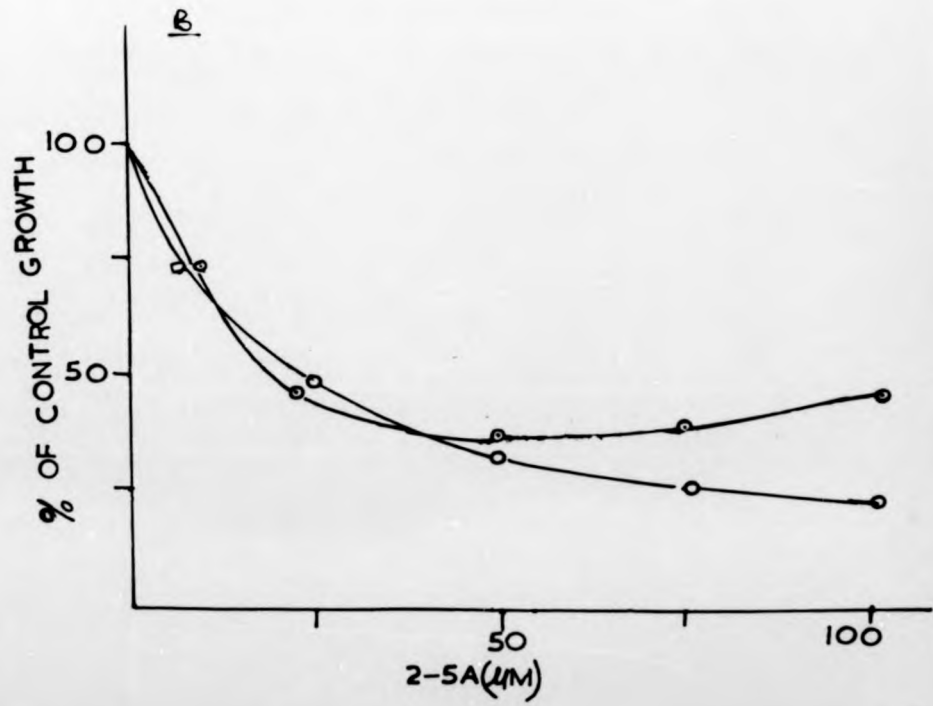
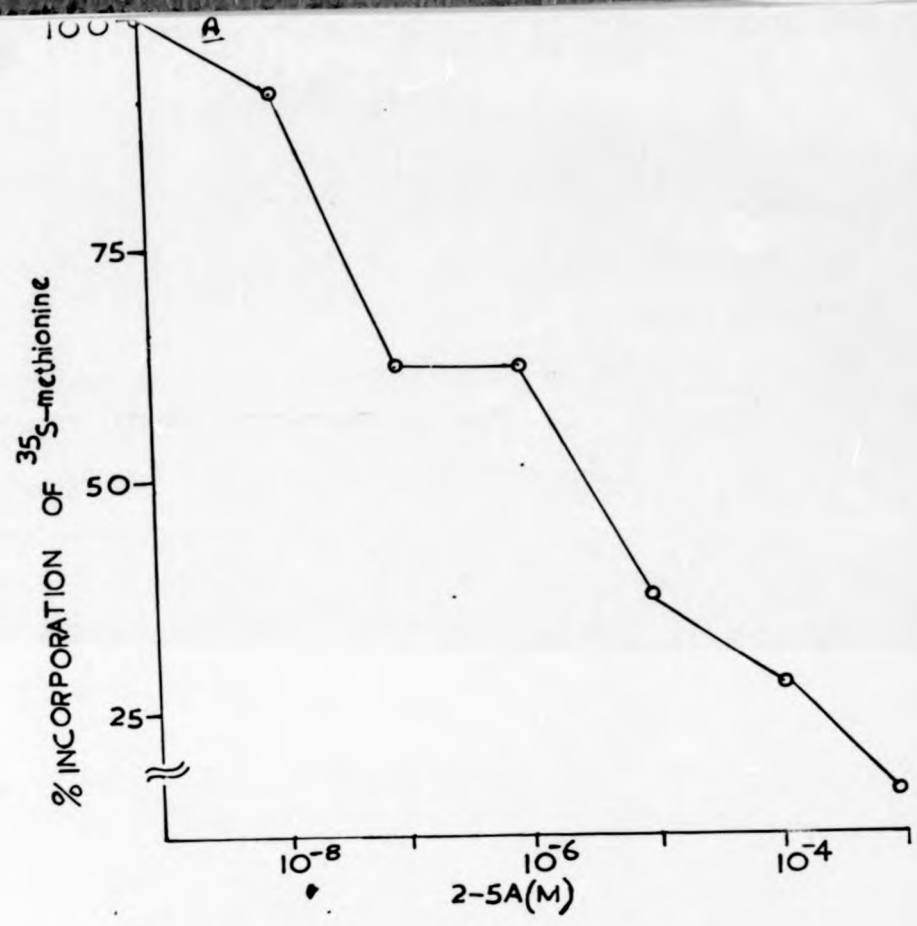


FIGURE 1.24 (A) Specific inhibitory activity of 2-5A on protein synthesis in vivo. See text for details. The total ^{35}S -methionine counts per min. incorporated into the permeabilised cells in the absence of 2-5A at various concentrations was determined. Incorporation of ^{35}S -methionine into permeable cells in the presence of different concentration of 2-5A is expressed as a % of the control value (no 2-5A).

(B) Effects of varying concentrations of 2-5A on the growth of ((o) MG63, and ⊙ HFF) cells. Cells (5×10^5 cells/plate) were cultured for 24 hours. Various concentrations of 2-5A were added to duplicate plates, and incubated for 48 hours, after which viable cells (excluding 0.1% trypan blue) were counted. The results are expressed as a % of control growth (cell count with 2-5A/cell count in control) $\times 100$.

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1.10.10.2 SPECIFIC INHIBITORY ACTION OF 2-5A ON PROTEIN

SYNTHESIS IN MG63 CELLS

Protein synthesis in MG63 cells could be inhibited by 2-5A when these cells were first permeabilised by incubation in hypertonic medium. When incorporation of ^{35}S -methionine into acid-insoluble material was estimated after 2 hours incubation period, it was found that an increase in concentration of 2-5A resulted in a decrease in incorporated radioactivity (Fig. 1.24A). It was also observed that exposure of the cells to the oligonucleotides for as little as 10 mins. before resealing by incubation in normal medium was sufficient for the subsequent inhibition to be observed. Protein synthesis was inhibited by 50% in the presence of concentration of 2-5A of about $1-0.5\ \mu\text{M}$ (following table and Fig. 1.24A). It had earlier been observed that the concentration of 2-5A required to give a 50% inhibition of cell-free rate synthesis was $0.3-1\ \text{nM}$ and that $10\ \text{nM}$ of 2-5A caused 50% inhibition of protein synthesis in vivo using BHK cells (Williams and Kerr, 1978). Concentration of inhibitor necessary to inhibit protein synthesis by 50% was found to vary slightly between experiments, despite the addition of 2-5A to cells during the hypertonic treatment as suggested by above authors.

The time course of incorporation of ^{35}S -methionine into the permeable cells treated with 2-5A at $100\ \mu\text{M}$ is shown in Fig. 1.23A. This inhibition was detectable within 10-30 mins. after resealing, and peaks at 1.5 hours, but is reversed on prolonged incubation.

The time required for the control levels of protein synthesis to be obtained depends on the concentration of 2-5A used (Fig. 1.33B).

Thus, both the rate and extent of incorporation are inhibited when 2-5A is included in the hypertonic medium. The table shows the effects of different 2'-5' linked oligoadenylic acids on protein synthesis in vivo.

Additions	Concentration (μ M)	%Inhibition of Incorporation of ³⁵ S-methionine
None	-	0
None	-	0
2-5A	100	84
	50	60
	1	50
'Core'	50	40
	1	18

Similar inhibitory property of the core has been reported by Kerr et al. (1978). Thus, the core was about 5 fold less active as 2-5A in BHK-21 cells. The table above shows a similar observation.

However, in vivo studies by Kerr et al. (1978), contrast with the in vitro observations by Kerr and Brown (1976). In the latter study, it was shown that the core exhibited no inhibitory activity in cell free system. To date, no direct evidence exists for the specific inhibition of viral or cellular mRNA synthesis and/or translation in vitro or in vivo, but

there is indirect evidence available. Because the synthesis of 2-5A led to degradation of mRNA via the activation of ribonuclease-F (Nilsen and Baglioni (1979)) it was suggested that the activation of endonuclease takes place near the replicate intermediates (RI) of RNA viruses. The RI then promotes synthesis of 2-5A in extracts of interferon treated cells. The RI are then degraded to a '20s' core which is resistant to digestion with RNases. This mechanism was proposed for the preferential degradation of dsRNA through action of 2-5A and thus 2-5A is thought to be responsible for discriminating between viral and cellular mRNA in interferon treated cells. This may well be one of the mechanism(s) responsible for the observed decrease in virus yield.

1.10.10.3 EFFECTS OF 2-5A ON CELL GROWTH

Effects on cell growth were assayed by counting viable cells. Triplicate cultures (5×10^4 cells per 35 mm culture dish) were treated with each of the various concentrations of 2-5A. Cells were counted by using 0.1% Trypan Blue for viability determination. Fig. 124B represents the dose-dependent growth inhibition of osteocarcinoma MG63 and HFF cells by 2-5A. While HFF shows a reduction of growth of 60% with 2-5A concentration 100 μ M, MG63 showed growth reduction of about 75% at the same concentration. The growth inhibitory action of 2-5A again is close to the observed inhibition by interferon of cell growth of osteosarcoma cells (Strander *et al.* 1973). Similar observations were made in other tumour cells including Hodgkins

lymphoma cells (Merigan, et al. 1978). Paucker, et al. (1962), and Lundgren, et al. (1979), have shown that interferon inhibits the proliferation of normal and malignant cells. Similarly, Creasy, et al. (1980), have shown that cells in phase G0-G1 states are vulnerable to the cytostatic action of interferon, and it was speculated that a specifically sensitive metabolic event may be the target of the cytostatic action of interferon. Specifically, the DNA synthesis events have been implicated. This points to a nucleic acid synthesis inhibition mechanism. Recently, 2-5A has been found to inhibit cell growth, and the core was similarly shown to inhibit DNA synthesis and cell growth in human lymphoblastoid (Daudi) cells (Williams and Kerr, 1980). It has also been shown in our study that 2-5A inhibits protein synthesis in MG63, and in addition the growth of HFF and MG63 cells were inhibited.

It is therefore possible that the protein and growth inhibitory actions of 2-5A we observed were due to prior inhibition of DNA synthesis. This possibility has also been suggested for the action of interferon (Creasy 1980). It is therefore likely that the action(s) of interferon is mediated by 2-5A. Table 6 shows that there is a slight reduction in virus yield from Semliki virus-infected cells permeabilised and treated with 2-5A. This observation was interesting in view of the known facts that in interferon-treated virus-infected cells, both the accumulation of viral RNA and viral protein synthesis may be inhibited (Friedman, 1977). It is therefore possible that either of these phenomena may be mediated by 2-5A.

However, it is also possible that all these events represent independent responses to the 2-5A system, either partially, or wholly unrelated to

TABLE 6

INHIBITION OF VIRUS GROWTH BY 2-5A

Treatment	Infection	SF Virus Yield	
		at 12 hours	at 24 hours
100 μ M 2-5A permeabilised cells	0.3	1.7×10^4	1.8×10^5
Control permeabilised cells	0.3	1.8×10^5	1.9×10^6
Control cells	0.3	1.2×10^3	2.0×10^6
100 μ M 2-5A permeabilised cells	30	2.0×10^6	1.3×10^6
Control permeabilised cells	30	5.2×10^7	1.5×10^6
Control cells	30	3.2×10^6	1.7×10^6

The results are averages of 2 determinations.

interferon action. However, partially, and tentatively, as mentioned earlier, Nilsen and Baglioni (1979) have shown that 2-5A system may be responsible for the discrimination between viral and host mRNA in interferon treated cells. There are still questions being asked as to why there has never been any observation involving the preferential degradation of either cellular mRNA (Clemens, and Williams, 1978), or viral mRNA (Williams, et al. 1979).

As yet no direct evidence has been provided for the 2-5A dependent endonuclease in interferon-treated virus infected cells, (Baglioni, 1979), nor has it been shown that cleavage of nascent RNA chains is responsible for the inhibition of virus replication in intact cells. However, localised 2-5A dependent endonuclease processes may explain discrimination between viral and cellular mRNA, and these may in turn be responsible for interferon action(s). In line with experimental evidence, wider roles (e.g. regulatory role) have been suggested for 2-5A in cells (Williams, and Kerr, 1980). In this study, the following have been achieved:

- (i) A contributory proof of the structure of 2-5A.
- (ii) Optimum conditions for the synthesis of the core by the phosphodiester approach and phosphorylation techniques to give 2-5A.
- (iii) Contributory proof of the biological functions of the 2-5A system.

It is therefore suggested from the above evidence that 2-5A discovered in interferon-treated cells, inhibits protein synthesis, and cell growth, and that the 2-5A may be responsible for inhibitory

action(s) of interferon. A wider significance for the 2-5A system (e.g. regulation of normal cell growth and development) is also suggested.

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NOTATION

CHAPTER TWO

Immobilised enzymes are active enzymes bound to insoluble matrices, proteins can be immobilised in a number of ways:

- (a) Trapped within gels or microcapsules.
- (b) Adsorbed tightly to insoluble materials (clays, starches, ion-exchange resins).
- (c) Copolymerised with some other repeating monomer.
- (d) Cross-linked with a bifunctional reagent around an insoluble matrix.
- (e) Derivatised by the addition of long polyamino chains until an insoluble material is produced.
- (f) Covalently attached to an insoluble carrier.

A protein can be covalently attached to an insoluble support, either by activating the support material for reactions with groups on the protein or by using a coupling reagent to link protein to matrix or the protein molecule may be activated for coupling to the support material (Zaborsky, 1973).

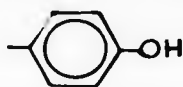
The conditions for coupling have to be chosen so that the protein remains as far as possible in its active conformation after the covalent coupling procedure. Protein molecules (enzymes, receptors, etc.) exist in their active forms only in a small number of active

FIGURE 2.1 Coupling of proteins to matrix: amino acid residue
on proteins that are amenable to covalent coupling
to a carrier.

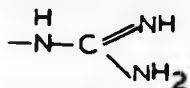
$-\text{NH}_2$ Amino of Lys and N terminal amino group

$-\text{SH}$ Sulphydryl of cysteine

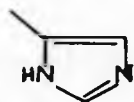
$-\text{COOH}$ Carboxyl of aspartate and glutamate and C-terminus carboxyl



Phenolic of tyrosine



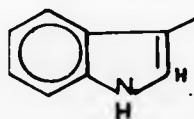
Guanidino of arginine



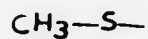
Imidazole of histidine



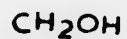
Disulphide of cystine



Indole of tryptophan



Thioester of methionine

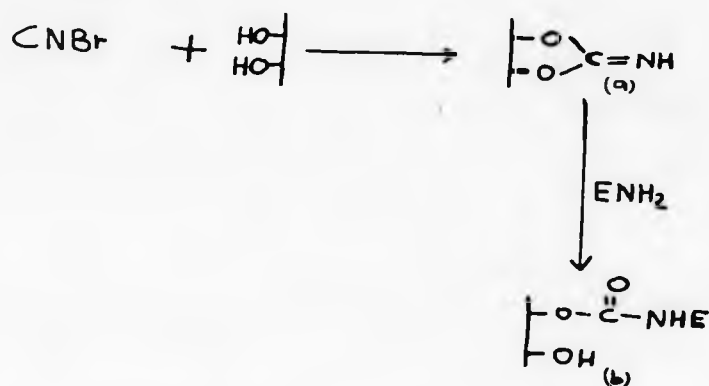


Hydroxy of serine. Threonine

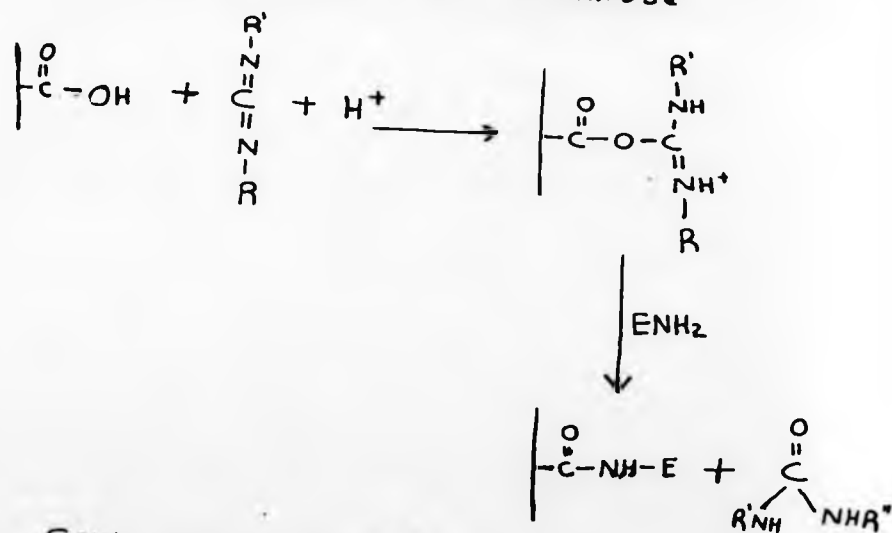
d residue
coupling

- FIGURE 2.2 (i) The activation of Sepharose with cyanogen bromide leads to the formation of a reactive imidocarbonate (a) in the Sepharose. The activated Sepharose will then couple to an amino group on the protein (ENH_2) to yield the immobilised product (b).
- (ii) Coupling of an enzyme to a carboxylic polymer via carbodiimide.
 - (iii) Coupling of an enzyme to carboxylic-polymer via diazonium salt.
 - (iv) Insolubilisation of enzyme by N-carboxylanhydrides in a copolymerisation process.
 - (v) Coupling of enzyme to acyl-azide-activated material.

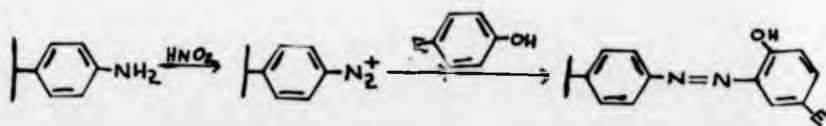
Fig. 2.2



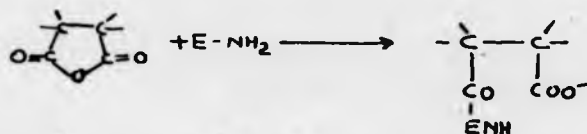
(i) CNBr activation of Sepharosa



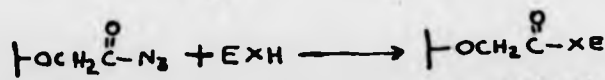
(ii) Carbodiimide Coupling



(iii) Linkage through Diazonium Salt



(iv) Use of Support containing anhydride groups.



(v) Linkage to acyl-azide-activated material

native conformations, which are determined by the protein's primary amino acid sequence. The bonds involved in maintaining its secondary, tertiary, and quaternary structures (except for the disulphide bond) are non-covalent ones. These non-covalent interactions are salt or hydrogen bonds and hydrophobic interactions; each of these as an individual bond is relatively labile, and it is the sum of a large number of these individually weak bonds that gives stability to the final structure of the protein. However, the fragile nature of each bond, limits the solution conditions under which a protein can maintain an active conformation. Thus the temperature might be kept below 35° and pH must be kept between 3 and 10.

Groups available for covalent attachment of proteins have been described by Srere and Uyeda (1976). The reactive groups are listed in Fig. 2.1.

Besides the nature of the side chains, the relative concentration of the amino residues in the protein molecule and the hydrophobic nature of the various amino acid residues are other important factors involved in the covalent attachment of a protein to a solid support. The energetic basis for the internalisation of the residue has been ascribed to the change in free energy (ΔG transfer) for the transfer of a residue from an aqueous to non-aqueous environment (Tanford 1962). The glutamine, asparagine, glycine, leucine and isoleucine, tyrosine, methionine, threonine and serine residues have positive values of ΔG and may thus take part in coupling reactions, but the negative ΔG transfer value for the amino acids tyrosine, methionine, threonine, and serine residues, mean the choices

of reaction with these amino acids are reduced, because it is more likely that these amino acids will be found inside the protein.

In general, most reactions of amino acids involved in coupling to carriers can be classified as carbonyl-type reactions with nucleophilic groups on the protein, -NH_2 , -SH and -OH (Jencks, 1969). The pH is one of the factors that determine the rate of nucleophilic reactions. Since the reactive species would be -NH_2 , -S^- , and -O^- , then at pH value of about 9, the concentration of -NH_2 residues would be highest of the three and therefore the most likely to react. In terms of nucleophilic reactivity, the S anion is generally one or two orders of magnitude larger than that of normal N and O anions of comparable basicity. However, the stability of the esters formed by these groups in nucleophilic reactions varies widely. Thioesters are much less stable than the oxygen esters; these in turn are less stable than the substituted amides that are formed. Considering the factors mentioned above - it is apparent that lysine would be predicted to be the most likely coupling residue, followed by cysteine, tyrosine, histidine, (aspartic and glutamic acids), arginine, tryptophan (serine and threonine), and methionine.

The main techniques for coupling proteins to a supporting material (Fig. 2.2) are:

- (a) Cyanogen bromide activation of polysaccharides (e.g. agarose). A reactive imidocarbonate is formed initially, which subsequently couples to an amino group on the protein to yield the immobilised product (Axen, *et al.* 1967).
Mainly the ϵ -amino group of lysine or the α -amino group

of the N-terminal amino acid are thought to be responsible for coupling.

- (b) Carbodiimide activation of polysaccharides. Water-soluble carbodiimides have been employed, at room temperature, *and* neutral pH to couple an enzyme to a carboxylic polymer via an amide bond. If the support contains amines or substituted amines, in the presence of carbodiimides the linkage would be through a carboxyl group on the protein.

- (c) Anhydrides have been found to react extremely rapidly with lysyl, cysteinyl, tyrosyl, and histidiny residues. This reaction can often be reversible. N-carbonyl anhydrides react specifically with lysyl residues and have been used to insolubilise enzymes by copolymerisation processes.

Acid azides have been used extensively to couple proteins to support. For example, activation of carboxymethyl cellulose can take place by esterification to yield methyl ester.

Hydrazinolysis of the ester followed by reaction of the hydrazine with nitrous acid forms acyl azide. The acyl azide reacts with the nucleophilic centres, e.g. sulphhydryl, amino, or hydroxyl groups, to yield the thioester, amide or ester linkage.

- (d) Celluloses have been activated by cyanuric chloride and its dichloro derivatives, isothiocyanate—containing supports have been used for the covalent coupling of proteins (Zaborsky, 1973).

- (e) Proteins have been coupled to p-benzoquinone activated Sepharose by Brandt, *et al.* (1975).

(f) Proteins have been coupled to inorganic supports, e.g. glass, via direct adsorption to glass, or activated glass. Proteins have been coupled to glass via activated glass derivatives including alkylamine, silanised and carboxy derivatives. Inorganic bridge formation between the glass and enzyme (via e.g. TiCl_4) has been used to insolubilise proteins (Weetall, 1976).

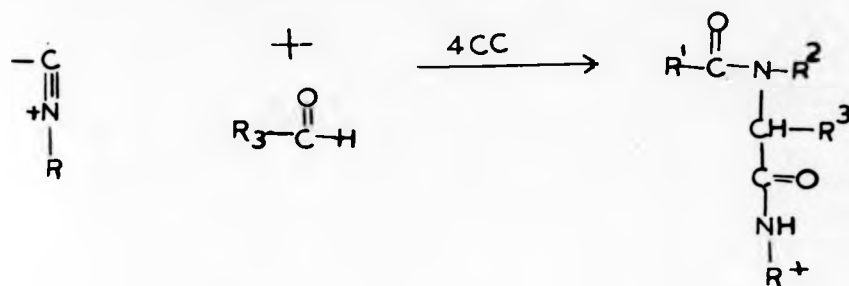
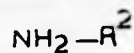
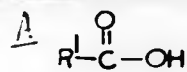
The immobilisation of enzymes on polysaccharides or other hydroxylic matrices may be accomplished by two different methods: covalent irreversible enzyme attachment and reversible enzyme attachment. Reversible coupling can be of two different types, either via covalent coupling (e.g. isocyanide method via introduction of carboxyl groups - Ugi (1962) - or adsorptive coupling (e.g. aliphatic and aromatic ether derivatives of agar as hydrophobic adsorbents for enzyme immobilisation). When reversible coupling is used, enzyme elution from the support can be corrected by further addition of enzyme. The ability to regenerate the system is its distinct advantage. Covalent irreversible coupling should preclude leakage of enzyme, but when catalytic activity is handicapped to an intolerably low level, the enzyme gel may not be easily regenerated. Examples of covalent irreversible coupling include imidocarbonate bond formation, due to the reaction between cyanogen halides with the hydroxyls of a polysaccharide matrix (Axen and Ernback, 1971).

Various synthetic carriers (e.g. hydrophilic cross-linked polyacrylic polymers, (Weston and Avrameas, 1971)), have been used for covalent attachment of enzymes. Similarly acrylic gel entrapment, is another method of covalent attachment, where an acrylic gel is

formed around an enzyme or the enzyme is introduced into a preformed polymer with subsequent reactions causing a cross-linked network to be formed, thus decreasing the enzyme's mobility (Hicks and Updike, 1966).

Nylon, a family of linear polymers consisting of repeating assemblies of methylene groups joined together by secondary amide linkages has been successfully used for immobilisation of enzymes. The linkage is often formed by either of two methods, covalent binding of enzymes to nylon by methods involving peptide bond cleavage (Hornby and Filippusson, 1970), or by covalent binding of the enzymes to nylon by methods involving O-alkylation of the nylon (Benson and Cairns, 1951). An alternative approach to covalent binding of enzymes to nylon is by a method involving N-alkylation of the nylon backbone. Chemically reactive side chain is generated either by mild acid hydrolysis (Sundaram and Hornby, 1970), this results in the exposure of $\text{COOH}\dots\text{NH}_2$ pairs on the surface of a nylon structure, or by resealing of the newly formed $\text{COOH}\dots\text{NH}_2$ pairs by a four component condensation reaction that involves, the neighbouring carboxyl, amino groups on the nylon backbone, an aldehyde, and an isocyanide (Vretbald and Axen, 1971). The four component condensation (4CC) is very interesting. In the 4CC between carboxyl, amine, aldehyde, and isocyanide (Figs. 2.3) the carboxyl and amino components (R^1 and R^2) combine to form an N-substituted amide, the aldehyde and isocyanide components (R^4 and R^3) appear as the side chain on the amide nitrogen. These reactions allow in principle for considerable versatility, since, by proper choice of aldehyde, and isocyanide, various functional groups can be

- FIGURE 2.3
- (A) Four component condensation reaction between amines, carboxyl, aldehyde, and isocyanide.
 - (B) Synthesis of polyisonitrile-nylon.

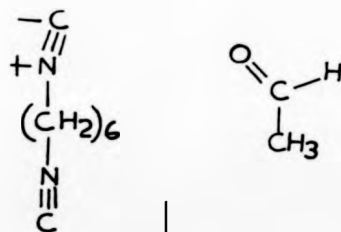
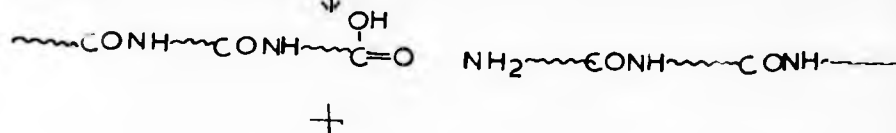


B

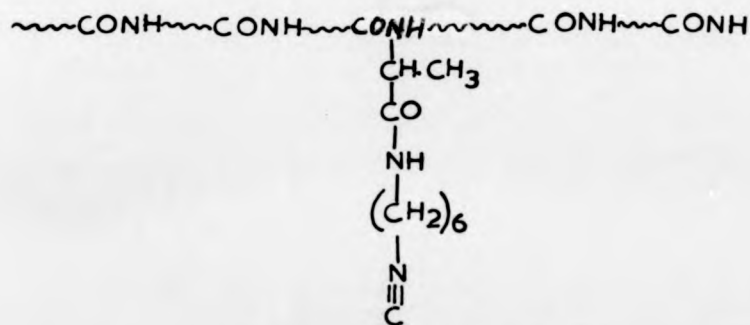


Nylon-6

Controlled hydrolysis



4CC



POLYISONITRILE-NYLON

introduced on the N-alkyl side chains of the reformed amide group of the nylon. An example of such a procedure is shown in Fig.2.38 and here, using acetaldehyde, or isobutyral as the aldehyde component and a bifunctional isocyanide, 1,6-diisocyanohexane, leads to nylon derivatives containing isocyanide (isonitrile) functional groups (Goldstein, et al. 1974). A final method of immobilising enzymes on synthetic supports, involves the use of inorganic support materials (Chin, et al. 1974). The characteristics of these supports are very important and they must meet certain criteria before they can be considered appropriate for use as immobilising supports (Eaton, 1974). Controlled pore glass and titanate have been used as supports, thus a stannous bridge has been used to couple urease to the former support (Messing, 1974) and in our studies, titanate bridge formation of phosphotransferase to controlled-pore glass has been achieved. Enzymes can be coupled to inorganic carriers either by the adsorption technique described earlier, or by covalent attachment methods. Any of the previously mentioned covalent methods can be easily adapted for use (e.g. carbodiimide coupling, Line, et al. 1971), and thiocyanate coupling (Weetall, 1976), except that for the best performances, the inorganic carrier has to be derivatised first before covalent coupling. Derivatives of inorganic carrier often used include alkylamine derivatives (Weetall, 1976). Preparation of carboxyl derivatives of inorganic carriers and coupling enzymes to the derivatives (via either carbodiimide or N-hydroxysuccinimide ester, or acid chloride) have been suggested as being preferable for use in both acidic and alkaline pH, since there will be less chance of cross-linking to the carboxyl-activated carrier as will likely occur

if enzymes were coupled to alkylamine-activated carrier (Weetall, 1976). However, it is also possible to couple an enzyme directly (i.e. without prior derivatisation) to an inorganic support (e.g. glass). This is often achieved by taking advantage of the silanol residues on the surface of glass, or the metal oxide groups on ceramic surfaces, which are capable of reaction with several organic activating groups. Thus, the activated carrier will interact with the enzymes, forming permanent linkages. In this regard, cyanogen bromide and the bifunctional reagent 4,4'-bis(2-methylene benzenediazonium) chloride have been suggested for coupling enzymes to inorganic supports (Weetall, 1976, and Messing, et al., 1974). In the absence of any of the above activating groups, aqueous or organic silanisation techniques have been suggested for direct (i.e. no prior derivatisation) covalent coupling of enzymes to inorganic supports. After optimising inorganic carriers, by pH, cofactors, pore diameters and preconditioning enzymes can be adsorbed directly to the inorganic supports (Messing, 1974).

Generally, among the large numbers of supports available for the immobilisation of enzymes, the synthetic carriers offer the best choice because of their good mechanical and chemical stabilities and inertness to microbial degradations.

The study of enzymes artificially bound to or within solid supports has attracted considerable attention owing to the potential of these water-insoluble preparations, as highly specific, reusable and removable reagents. Thus, immobilised L-amino acylase was used for the commercial separation of racemic mixtures of amino acids

(Tosa, et al. 1967). Immobilised enzymes have promising uses as industrial catalysts to convert sucrose into fructose (Poulsen and Zittan, 1976).

Potential uses of immobilised enzymes in the general biomedical areas are manifold and includes: therapy, clinical analysis, preventive and environmental medicine, and biochemical and biophysical research (Chang, 1980, and 1964). Their potential also lies in their ability to discriminate between various functional groups in a molecule (Ademola and Hutchinson, in press). Because of their being reusable and possessing increased stability, immobilised enzymes as reagents and catalysts with these two properties are receiving considerable attention from the rapidly increasing number of chemists involved in the synthesis of asymmetric and medicinal products (Scott and Valentine, 1974).

Our investigations are mostly concerned with the development and use of immobilised phosphotransferase for the specific phosphorylation of the potentially useful metabolite, 2-5A oligonucleotide.

Following the successful purification of the wheat shoot phosphotransferase by conventional and affinity techniques (Ademola and Hutchinson, 1980), the enzyme was immobilised on various organic and inorganic supports, and utilised in the form of a packed bed reactor, for the phosphorylation of the 5'-hydroxy of 2-5A linked oligonucleotide.

Nucleoside phosphotransferase (E.C. 2.7.1.77) was purified from carrot (Brunngraber and Chargaff, 1967). Harvey, et al. (1970) have isolated a similar enzyme from wheat shoots, and without further purification, the wheat shoot enzyme was then used to prepare 5'-nucleotides and analogues on a small scale. A number of workers have used the phosphotransferase, with suitable donors, for the conversion of unprotected nucleosides into nucleoside 5'-phosphates, based on the relatively low specificity of these enzymes as regards the aglycone. The procedure is particularly useful for acid- or alkali-labile nucleosides, for rare or radioactively labelled nucleosides (where recovery of material is important) and in the case of the wheat shoot enzyme, for the phosphorylation of the primary hydroxyl groups of nucleosides in which the sugar residue is not furanose (Giziewicz and Shugar, 1975). The phosphotransferases catalyse the phosphate transfer from a suitable donor such as a 3' or 5'-mononucleotide to a nucleoside acceptor forming a new 5'-nucleotide. In the absence of a suitable acceptor, the phosphate is released as inorganic phosphate. A second independent hydrolytic function is also present. The transfer function is assayed by determining the amount of 5'-nucleotide formed when the enzyme is incubated with a nucleoside and a suitable phosphate donor. p-Nitrophenyl phosphate has been found to be a useful phosphate donor and has the advantage of providing a visible indicator with the formation of the yellow p-nitrophenol. Accurate measurement of transferase function is often

determined by separating the enzymatically formed nucleotide which is measured spectrophotometrically.

EXPERIMENTAL

2.3 MATERIALS AND METHODS

2.3.1 MATERIALS

Commercial preparations of nucleosides and nucleotides were used without further purification. Phenyl phosphate as the disodium salt (Eastman Kodak) was recrystallised as described by Salmon, et al. (1964).

p-Nitrophenyl phosphate and ethyl phosphate were used as their disodium salts. 2,4-Dinitrophenyl phosphate was prepared by the method of Ramirez (1966). Proteins used as molecular weight markers were obtained from Sigma. Other chemicals were of reagent grade of the highest quality available.

Analytical Procedures

Phosphorus was determined colorimetrically (King, 1932), and p-nitrophenol by colorimetry (Bray and Thorpe, 1954) or spectrophotometry (Brawerman and Chargaff, 1954). Nucleotide products were estimated by following the reaction with t.l.c. using F₂₅₄ cellulose on aluminium sheets developed with solvent A. After development, the regions which contained u.v. absorbing material were removed, the cellulose extracted with water (2 ml) and the nucleotide

content determined spectrophotometrically. Oligonucleotides were separated by solvent B. Protein was determined by the method of Lowry, et al. (1951) or occasionally by spectrophotometry (Kalckar, 1947).

2.3.2 ENZYME ISOLATION

(i) Enzyme Preparation

Etiolated wheat shoots (6 g) were harvested after 5 days (5" height) and were chopped (cold room, with scissors), and ground up in a pestle and mortar (4°) with sodium acetate pH 5.0 (0.02 M, 15 ml). The product was strained with muslin, and spun at 16,000 rev/min. for 10 mins. The supernatant was stored (at -10° in 10 x 20 ml or at -20° in 5 x 1.5 ml) in screw-topped vials. The amount of protein obtained at this stage = 25 mg/ml, protein was determined by the method of Lowry et al. (1951). The fractions were considered the crude extracts and referred to as fraction I.

(ii) Enzyme Purification

Step I

Ammonium sulphate fractionation: In this procedure, the temperature was maintained between 0-4°, crystalline ammonium sulphate was added gradually to the crude extracts to 35% saturation and stirred for 30 min. to 45 min., the precipitate then being removed by centrifugation and discarded as it was found to contain little or no transferase activity. Additional ammonium sulphate was added till 90% saturation was reached. During this procedure pH 5.0 was maintained. The mixture was left overnight and centrifuged

at 16,000g for 20 min. The precipitate was dissolved in buffer pH 5.0 (20 ml) and applied to a column of DEAE cellulose (20 g).

Step II

DEAE cellulose (20 g) column (0.5 x 50 cm) pre-equilibrated with sodium acetate pH 5.0 (0.05 M), was loaded with the enzyme and washed with sodium acetate pH 5.0 (0.05 M, 200 ml) until O.D.280 was 0.1 and then eluted with gradient of 0.1 M - 0.5 M sodium acetate pH 5.0. Fractions (10 ml/hour) were collected, the transferase activity was eluted after 8 hours. The major peak of the transferase activity was pooled.

Ammonium sulphate fractionation II

The pooled fractions were subjected to a second ammonium sulphate fractionation by addition of ammonium sulphate to 95% saturation. The mixture was left for 6 hours, and centrifuged at 16,000 for 15 min. The sediment was suspended in distilled water (20 ml) and dialysed against water.

DEAE Sephadex chromatography

The dialysed fractions were concentrated (freeze drying, vacuum dialysis or sucrose concentration), and applied to a column (0.5 x 30 cm) pre-equilibrated with sodium acetate pH 5.0 (0.04 M). The column was washed with the same buffer until the O.D.280 reached 0.1, and a pH gradient was used for subsequent elution of the transferase activity. The peak phosphotransferase activity was eluted at about pH 4.5. This peak was subsequently dialysed overnight against distilled water and concentrated by usual method.

Sephadex G-100 Column

The fractions above were concentrated as described and dissolved in water (2.0 ml), the enzyme fraction containing 8 μ g of protein was applied to a Sephadex G-100 column (1.5 x 45 cm), previously equilibrated with 0.05 M sodium acetate, pH 5.0. The enzyme was eluted with this buffer at a flow rate of 20 ml/hour, 3 ml fractions being collected. The active fractions collected after the assay were dialysed against sodium acetate pH 5.0 (0.05 M) and stored in a frozen state at -20° . Several enzyme preparations made in this manner were used in all subsequent studies.

2.3.3 AFFINITY CHROMATOGRAPHY

Preparation of active phosphotransferase by affinity chromatography on Matrex TM Gel Blue A

(i) Preparation of Gel Suspension

Gel suspension was mixed by gentle stirring, and taken (2 ml) out of the sample bottle with a syringe. The gel was washed in a sintered funnel with starting buffer (5 x 4 ml).

(ii) Analytical Method of Protein Purification

The gel was equilibrated after pouring into a short column (0.6 x 10 cm), with starting buffer (10 ml). The enzyme samples (i.e. samples from either fractions 1-6 above) were added, and the effluents (2 ml) were collected on a Whatman paper, 2 separate drops of the effluent were collected on the paper for every portion. After the coupling buffer, sodium acetate pH 5.0 (0.05 M, 10 ml) was

added, the eluant buffer (sodium acetate, pH 5.0, 0.2 M) was added to elute the protein bound. When appropriate as indicated, a gradient of the eluting buffer was used. Portions of the eluting buffer (10 x 1 ml) were added and drops were collected for appropriate assay. The papers were dried, cut into halves lengthwise, and one half stained for protein, and the other half was cut into appropriate sizes and incubated for assay of phosphotransferase activity. This technique, which was adapted from Chelsea Medical Science Course, was found useful and convenient for continuous assay of the enzymes eluting from the column. The papers were stained for protein using bromophenol, blue colouration indicated the presence of protein. Fractions lacking proteins were not assayed for phosphotransferase or phosphatase activities. All fractions which showed protein present had their duplicates assayed quantitatively for protein and phosphotransferase activities. To establish optimum conditions for binding and elution of the phosphotransferase activity, the concentration, pH of the buffer, temperatures, and column sizes were varied.

Preparatively, the column size was (1.5 x 40 cm) of Matrex Gel blue A, coupling buffer, sodium acetate pH 4.0 (0.05 M) and eluting buffer, sodium acetate, pH 5.0 (0.05 M) or uridine (50 mM), in sodium acetate pH 5.0 (0.05 M). All operations were conducted at cold room temperature. Flow rate was 15 ml/hour.

2.3.4 ENZYME ASSAY

Soluble Enzyme

The eluates from the various columns were tested for enzyme

activities as described above. Fractions showing promising enzymic activities were assayed as follows.

(i) Phosphotransferase - In a volume of 1.0 ml of sodium acetate pH 5.0 (0.1 M), p-nitrophenyl phosphate (100 mM) oligonucleotide or adenosine (200 mM), and enzyme were incubated at 37°C for 1 hour, the reaction was stopped with sodium hydroxide (0.1 M, 0.1 ml). Portions (0.1 ml) from the whole mixture served for determination of nucleotide formed.

In all experiments the amount of enzyme required was determined by preliminary assays in order to establish optimum conditions for phosphorylation of nucleoside and oligonucleotide.

For kinetic studies, the incubation mixture was as discussed above, except that each reaction was stopped after 30 min. and the amount of enzyme required was determined by preliminary assays to establish that no more than 10% of substrates were transformed and that the products formed were directly proportional to the enzyme concentrations.

Immobilised Enzyme Assay

A crude measurement of the protein content was made by measuring the amount of unbound protein in the washings. A more accurate value was obtained by acid hydrolysis in 6 N hydrochloric acid at 110°C followed by estimation of amino acids with ninhydrin (Kay, et al. 1956).

A sample of immobilised enzyme was placed in a hydrolysis tube and 6 M hydrochloric acid was added. The tube was sealed and heated at 110°C for 4 hours. A standard was prepared by hydrolysing a known

amount of enzyme under similar conditions. The samples were cooled and filtered, the filtrates evaporated to dryness and made up to 10 ml with water. Aliquots (1 ml) were taken and citrate, pH 5.0 (0.02 M, 0.50 ml) ninhydrin (5% w/v in 2-methoxyethanol (0.20 ml)) and potassium cyanide (0.1 M) in 2-methoxyethanol (2%, 1 ml) were added. The samples were heated at 100° for 15 min. and then cooled rapidly. If necessary, the samples were diluted with ethanol (6%) before measuring the absorbance at 570 nm. A standard curve was prepared at the same time using a known enzyme sample.

Phosphatase Assay

Each assay tube contained, in a total volume of 1 ml, sodium acetate pH 5.0 (0.1 ml), a suitable quantity of enzyme, and p-nitrophenyl phosphate (100 mM), with or without inhibitor (adenosine 2-50 mM). The samples were incubated (1 hour at 37°), the reaction was stopped by the addition of sodium hydroxide (1 M, 0.2 ml), and p-nitrophenol was estimated (Martin, T. Personal Communication), or assayed by the release of inorganic phosphate (King, 1932).

In the latter case, aliquots were removed from the reaction mixture (0.1 ml), and filtered, made up to 1 ml with hydrochloric acid (1.0 ml) and added to phosphate reagent (0.1 ml). The phosphate reagent consisted of one volume ascorbic acid (10%, w/v), water (2 vols.), ammonium molybdate (2.5%, 1 vol.) and sulphuric acid (6 N, 1 vol.). Samples were incubated for 20 min. at 45° and O.D. 820 nm was estimated. Initial velocities were taken from linear portions of the progress curves. Reciprocals of the initial velocities were plotted against reciprocals of substrate concentration

to determine the Michaelis constant. The kinetic constants were determined from initial velocity and substrate concentration data pairs. Values of k_m and v_{max} were obtained by the method of least squares (Morton, 1955).

2.3.5 EFFECTS OF pH ON ENZYME ACTION

These experiments, which all utilised the enzyme eluted from a Sephadex G-100 column, were also used to establish optimum conditions for affinity chromatography and immobilisation, and also for kinetic studies of both free and immobilised enzymes. The experiments were all carried out in similar fashion. The following buffers (Dawson, 1969) were used: glycine-HCl pH 3.6 (0.2 M); sodium acetate-acetic acid, pH 4.0 and pH 4.5 (0.2 M), sodium hydrogen maleate-sodium hydroxide pH 5.0, pH 5.6, pH 6.4 and pH 6.8 (0.2 M), tris-HCl, pH 7.2, pH 7.6, pH 8.0 (0.2 M).

For the study of the effects on phosphatase activity, 2-15 mM phosphate donor in buffer (0.1 M final concentration) was incubated at 37° (60 min.). The effects on transferase activity were followed with (a) 2-15 mM p-nitrophenyl phosphate and adenosine (25 mM) in buffer (0.1 M, final concentration), for 60min. at 37°, and (b) 25 mM p-nitrophenyl phosphate and 1-10 mM adenosine in buffer (0.1 M, final concentration), for 20 min. at 37°. In all other experiments (determination of required enzyme concentrations, termination of enzyme action, estimation of reaction products), the procedures described in the preceding section were used.

Detection of Ribonuclease Activity in Purified Enzyme (Kimhi, and Littauer, 1968)

A sample of enzyme was incubated in the presence of [14 C]-poly A in polymerisation buffer for eight hours at 37°. An aliquot was applied

to a cellulose t.l.c. plate which was developed in 1 M ammonium acetate:ethanol. The plate was cut into strips and radioactive products were detected by scintillation procedure.

2.3.6 IMMOBILISATION OF PHOSPHOTRANSFERASE ON INSOLUBLE SUPPORT MATERIALS AND USES

The immobilisation was carried out using different methods with soluble enzyme (concentration of 1 mg/ml). The amounts of protein bound, pH optima, enzymic activity were all determined in separate experiments. The immobilised enzyme was assayed as described above.

After the coupling of the enzyme to the support, the suspension was packed in a column and washed with 0.10 M sodium bicarbonate (200 ml), water (200 ml), and tris, pH 8.0 (0.10 M) and sodium chloride (1.0 M) (250 ml) and finally with sodium acetate pH 5.0 (0.05 M). The conjugate was stored at -4° in sodium acetate, pH 5.0 (0.05 M).

(a) Coupling to p-benzoquinone-activated Sepharose 4B

This was carried out as described by Brandt, et al. 1975). Sepharose 4B was swollen in appropriate buffer and mixed with ethanol containing benzoquinone to give a final concentration of 20% ethanol and 50 mM benzoquinone. For pH 3-4, 0.01 M sodium formate buffer was used for pH 4-6, 0.1 M sodium acetate, pH 6-8, 0.1 M sodium phosphate and pH 8-12, 0.1 M sodium bicarbonate. The activation was allowed to proceed at room temperature for 1 hour in phosphate buffer, pH 8.0, unless otherwise stated. After activation the gel samples were washed on a glass filter successively with 20% ethanol, water, 1 M NaCl,

and finally with water.

Activated gel samples (3 ml) were suspended in the appropriate buffer (3 ml) mentioned above under activation. Usually sodium acetate buffer pH 5.0 (0.1 M) was used and the protein was dissolved in the buffer before it was mixed with the activated gel. The coupling reaction was performed in closed, slowly rotating test tubes at 4°C usually for 24 hours. The products were then washed firstly with water in a glass filter and then extensively in a small column with 1 M KCl/0.1 M sodium bicarbonate buffer, pH 8.5 (24 hours), 1 M KCl/0.1 M sodium acetate pH 5.0 (24 hours), and finally with water. Gel samples used for determination of protein content were dehydrated by washing with acetone and then dried by heating at 70°C for 24 hours. The maximum amount (80%) of protein bound after 15 min.

(b) Coupling to Cyanogen Bromide Activated Sepharose 4B

Cyanogen bromide-activated Sepharose was either obtained commercially (Pharmacia C.B. Ltd., Hounslow, Middlesex, U.K.) or was prepared immediately before coupling using 50 mg cyanogen bromide per ml Sepharose. The coupling was carried out as described by Porath, et al. (Porath, et al. 1973). The gel spheres were washed with distilled water and the interstitial water removed on a sintered funnel. The 4% gel was suspended in cold (5°C) potassium phosphate buffer (5 mM, 10 ml) and the suspension was diluted with distilled water to a total volume of 20 ml. Cyanogen bromide solution (4.0 ml) was added in small portions during 3 min. The solution was stirred gently and the temperature was maintained at 5-10°C for a reaction time of 10 min.

(which includes the time required for the addition of the reagent). The product was washed on a glass filter with cold distilled water until neutral.

The activated gel was rapidly washed with sodium hydrogen carbonate solution (0.25 M, pH 9.0) and the gel (1.0 g) was transferred to a cylindrical reaction vessel. When sodium bicarbonate (0.25 M) was added, followed by the enzyme (6 mg) the coupling was performed by rotating the vessel end-over-end at room temperature for 24 hours. The products were washed in a small column connected to a water jacket.

(c) Attachment to Controlled Pore Glass

(i) The adsorption of the phosphotransferase to CPG-10 controlled pore glass (BDH Chemicals Ltd., Poole, Dorset, U.K.) mean pore size 85Å, surface 175 m²/g was carried out, as described by Messing (Messing, 1976) over 2.8 hours; the amount of protein bound was 60%.

The immobilised enzymes were prepared by exposing the carrier (1 g) to excess of the enzyme (30 mg) in buffered solution for 2 hours in a shaken water bath at 37°. The enzyme solutions were further allowed to react with the carrier at room temperature for 22 hours without shaking. The enzyme solutions were then decanted, and the immobilised preparations were washed, successively with 0.5 M NaCl and distilled water. The immobilised enzyme was then transferred for use.

(ii) With titanium tetrachloride. This was as described by Messing (1976). CPG (10 g) was transferred to a solution of titanous chloride (20%, 50 ml), mixed well, and placed in a desiccator

(which includes the time required for the addition of the reagent). The product was washed on a glass filter with cold distilled water until neutral.

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(ii) With titanium tetrachloride. This was as described by Messing (1976). CPG (10 g) was transferred to a solution of titanous chloride (20%, 50 ml), mixed well, and placed in a desiccator

containing sodium hydroxide. The desiccator was evacuated and placed in an incubator at 40°. After drying, the treated carrier was washed (3 X) with sodium acetate, pH 5.0 (0.05 M). The activated carrier was then added to the enzyme solution, which was composed of the above buffer and contained protein (10 mg) having a specific activity of (30 u/mg). The enzyme and carrier were stirred at 2° for 18 hours, the suspension was then centrifuged, and the immobilised enzyme was washed first with sodium acetate pH 5.0 (0.02 M) containing sodium chloride (1 M), then with the acetate buffer without sodium chloride. The immobilised enzyme was resuspended in the same buffer and stored at 4°. The preparation had an activity of 1200 units/g of solid.

(iii) Coupling to carboxyl derivatised CPG-10. To alkylamine CPG-10 suspended in sodium phosphate buffer pH 6.0 (0.05 M, 25 ml) was added succinic anhydride (0.1 g) and the suspension was stirred at room temperature for 24 hours with the pH being maintained at 6.0. The derivatised CPG-10 was filtered off, washed and dried in vacuo. The derivatised CPG-10 was activated with excess 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulphonate at pH 5.0 before the addition of the buffered enzyme solution at 1 mg/ml concentration. The amount of protein bound was 45% after 5 hours.

(iv) Coupling to alkylamine derivatised CPG-10. This enzyme was coupled directly to alkylamine CPG-10, using 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulphonate for 1 hour when 70% of the protein was bound. The alkylamine derivative was prepared as follows. To clean CPG-10 (1 g) was added distilled water (18 ml) plus γ -amino propyltriethoxy silane (10% w/v, 2 ml). After the addition of the

silane solution, the pH was adjusted to pH 4.0 with hydrochloric acid (6 M). The neutralisation of the basic silane solution caused heat to be produced. After pH adjustment, the reactants were placed in 75° water bath for 2 hours, filtered on a sintered glass funnel, and washed with distilled water (20 ml). It was later dried in an oven at 115° for 11 hours.

The enzyme was coupled by use of 1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimide, metho-p-toluene sulphonate. The enzyme (10 mg per gram carrier) was added directly to the mixtures, and left overnight. The product was subsequently washed and used.

Preparation of 5'AMP using immobilised phosphotransferase

Adenosine (100 mg) and p-nitrophenylphosphate (200 mM) in 0.1 M buffer (2 ml) were circulated continuously for 5 hours through the immobilised phosphotransferase (1.0 ml bed volume) in a water jacketed column. Alternatively, the incubation was performed in a container, which was sealed and kept at 37° for 5 hours before removal of solvents under vacuum and a portion of the product was dissolved in minimum amount of water and applied to cellulose t.l.c. which was developed in solvent D. The product was obtained in quantitative yield and moved with R_f which was identical to the R_f of authentic 5'AMP. The product was eluted from the cellulose with 0.55 M triethylammonium bicarbonate, lyophilised and identified as discussed earlier.

Preparation of phosphorylated oligonucleotides

The incubation mixture contained the same amounts as described above. After 5 hours incubation, the product of the reaction was applied to a column of Sephadex A-25 (1.5 x 30 cm) which had been equilibrated with 0.5 M NH_4HCO_3 at 4°. The oligonucleotides were

silane solution, the pH was adjusted to pH 4.0 with hydrochloric acid (6 M). The neutralisation of the basic silane solution caused heat to be produced. After pH adjustment, the reactants were placed in 75° water bath for 2 hours, filtered on a sintered glass funnel, and washed with distilled water (20 ml). It was later dried in an oven at 115° for 11 hours.

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eluted with a gradient of triethylammonium bicarbonate (0-0.4 M, pH 8). Oligonucleotides were characterised by phosphate analysis, thin layer chromatography and enzymic digestion as described in Section 1.9.3. Some characteristics of the immobilised enzyme are shown in Figs. 2.4-2.6.

2.4 RESULTS AND DISCUSSION

2.4.1 PURIFICATION OF PHOSPHOTRANSFERASE

The purification of phosphotransferase from wheat shoot and carrot sources by conventional methods has been adopted from the procedure of Brunngraber and Chargaff (1967). 248 g/l of ammonium sulphate was found to precipitate the protein instead of 216 g/l used by the author. DEAE Sephadex produced better resolution of the various proteins than DEAE cellulose, probably due to charge density variations and fractionation due to size and charge in the former case.

The major peak eluted from the column chromatogram was concentrated with sucrose or equilibrium dialysis. Only the peaks with enzyme activity > 100 units/ml each were collected. The enzyme preparation in the case of wheat shoot, was adapted from the procedure of Martin, T. (personal communication) and the crude supernatant was stored at -10°C in screw-topped vials.

A second DEAE-Sephadex purification and ammonium sulphate fractionation, with Sephadex G-100 column purifications were included routinely in contrast to the original technique used for the carrot enzyme. The DEAE treatment helps to remove nucleic acid from crude preparation

of phosphotransferase (Thanassi and Singer, 1966). All these steps were found to remove considerable amounts of phosphatase, as their inclusion at least in the wheat shoot purification, produced enzymes which have improved transferase activities.

Earlier purifications of the carrot enzyme have included acetone 30-70% precipitation, raising the specific activity by a mere factor of 1.3, with loss in yield of 10% (Strider, et al. 1968). This step was therefore excluded in this work.

The results obtained during the purification of the wheat shoot enzyme using conventional techniques, agreed closely with the results obtained by Brunngraber and Chargaff (1967) during the purification of the carrot enzyme.

In both the carrot and wheat shoot enzyme prepared, fractions 6 were suitable for phosphorylation of oligonucleotides.

The purification of phosphotransferase by conventional methods mentioned above, is a lengthy procedure during which the enzyme can be degraded by proteolytic enzymes or denatured during handling. Affinity chromatographic purification of the enzyme as performed, was found to reduce time, it simplified the purification and minimised denaturation of the enzyme. Immobilised derivatives of reactive triazonyl dyes have been prepared and covalently linked to 5% cross-linked agarose, and these have found extensive uses as group selective affinity chromatographic media in the purification of enzymes requiring as cofactors NAD^+ and NADP^+ . Before this development, blue dye agarose conjugates have provided a simple method for fractionation of both NADP^+ -dependent and non-dependent enzymes,

although the use was first thought to be limited to NADP enzymes.

We have found that the Matrex Gel Blue can be used for the purification of phosphotransferase. In a typical experiment, the dyes were pretreated as described by the manufacturers and modified as described above. A column (0.3 x 40 cm) was washed with 2-4 bed volume of urea (6 M), sodium hydroxide (0.6 M) followed by 20 bed volume of starting buffer. The elution profile is shown in Fig. 2.5(B). Table 2.1 shows the yield of 5'AMP obtained when the Cibacron purified enzyme was used for nucleotide synthesis. If the binding of the enzyme to the ligand is anything near biospecificity, it should be possible to elute the enzyme with a pulse of substrate. This has been achieved using uridine (50 mM).

2.4.2 EFFECTS OF pH AND TEMPERATURE ON THE BINDING OF THE ENZYME TO THE MATRIX

The results of experiments with the phosphotransferase are shown in Fig. 2.6A. The binding of the enzyme to the matrex gel is strongest at pH 5.0-6.0 with a sharp drop on either side of the optimum. The binding of the enzyme is strongest when the column is operated below 5°, the binding being 50% of maximum at 20°. The fact that binding is temperature sensitive indicates that the binding to the ligand is not an ion exchange effect but biospecific. Above 20°, leaching of the dye and enzyme leakage occurred.

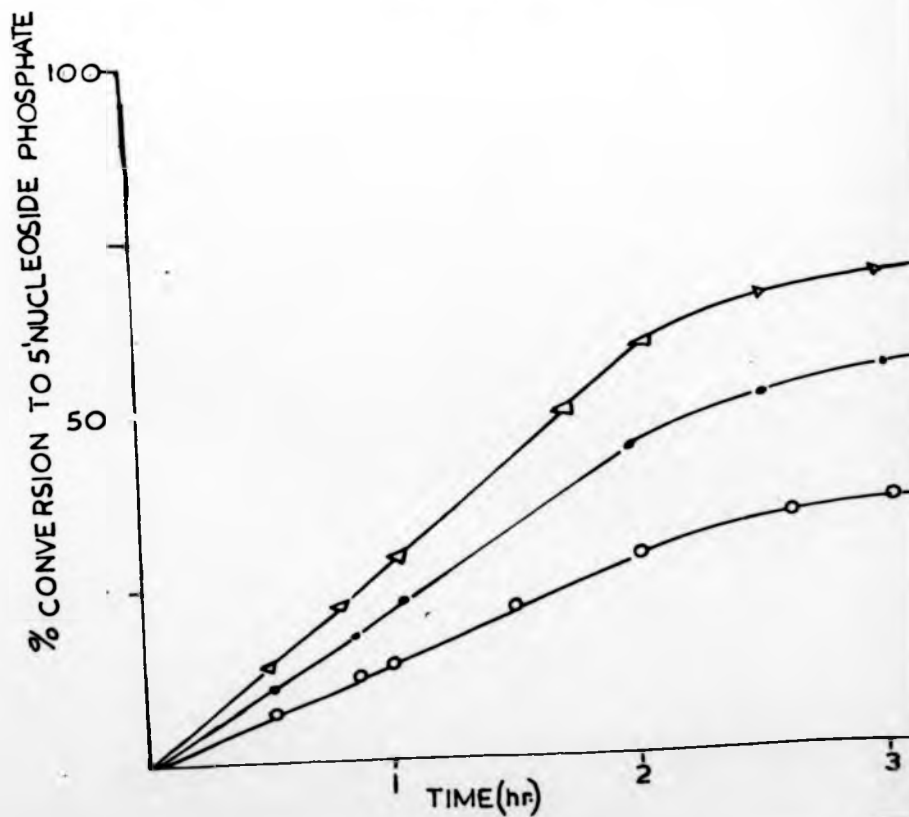
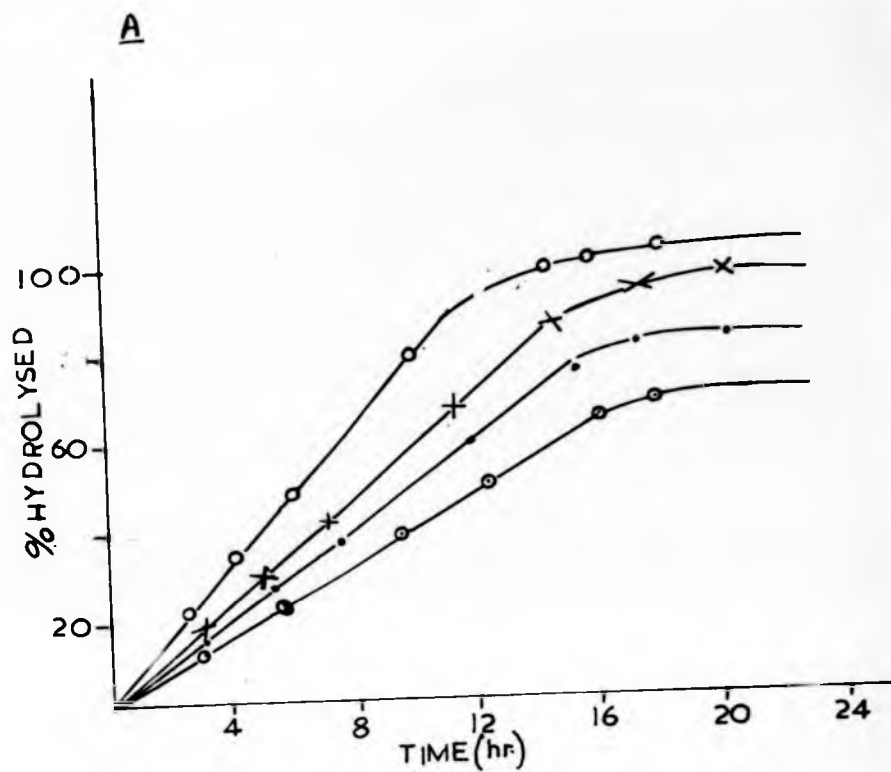
The binding capacity of the dye as determined under optimum pH and temperature was 10 mg/ml dye.

FIGURE 2.4

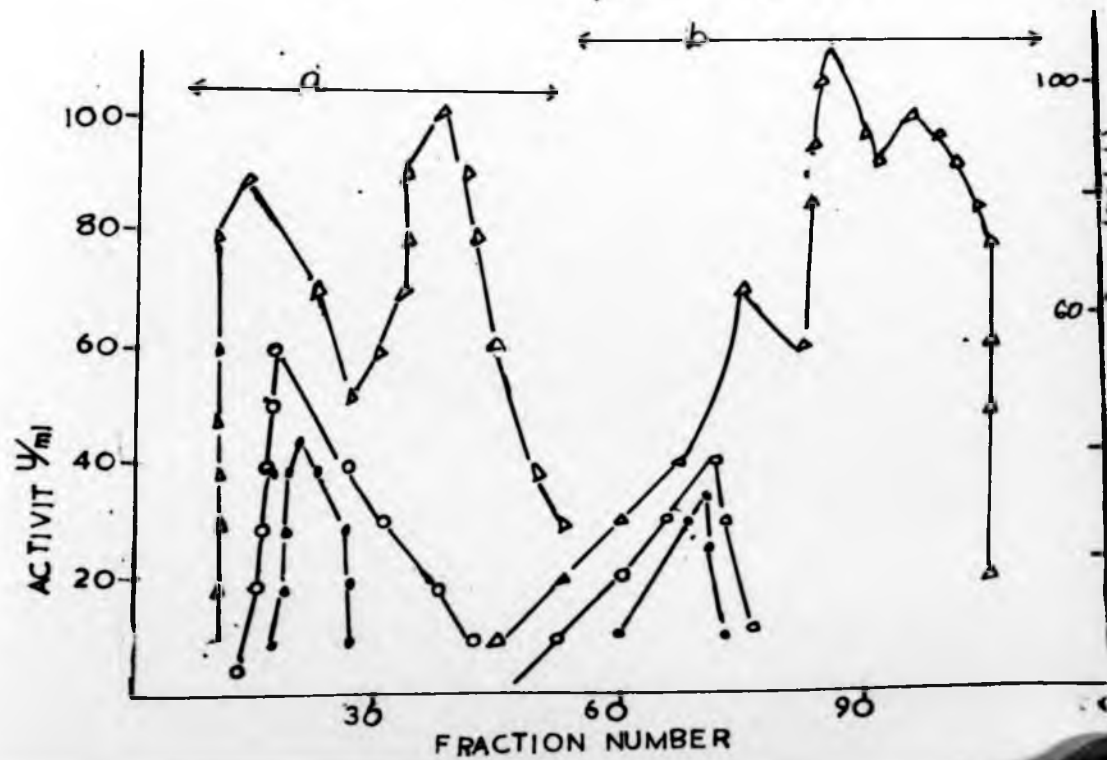
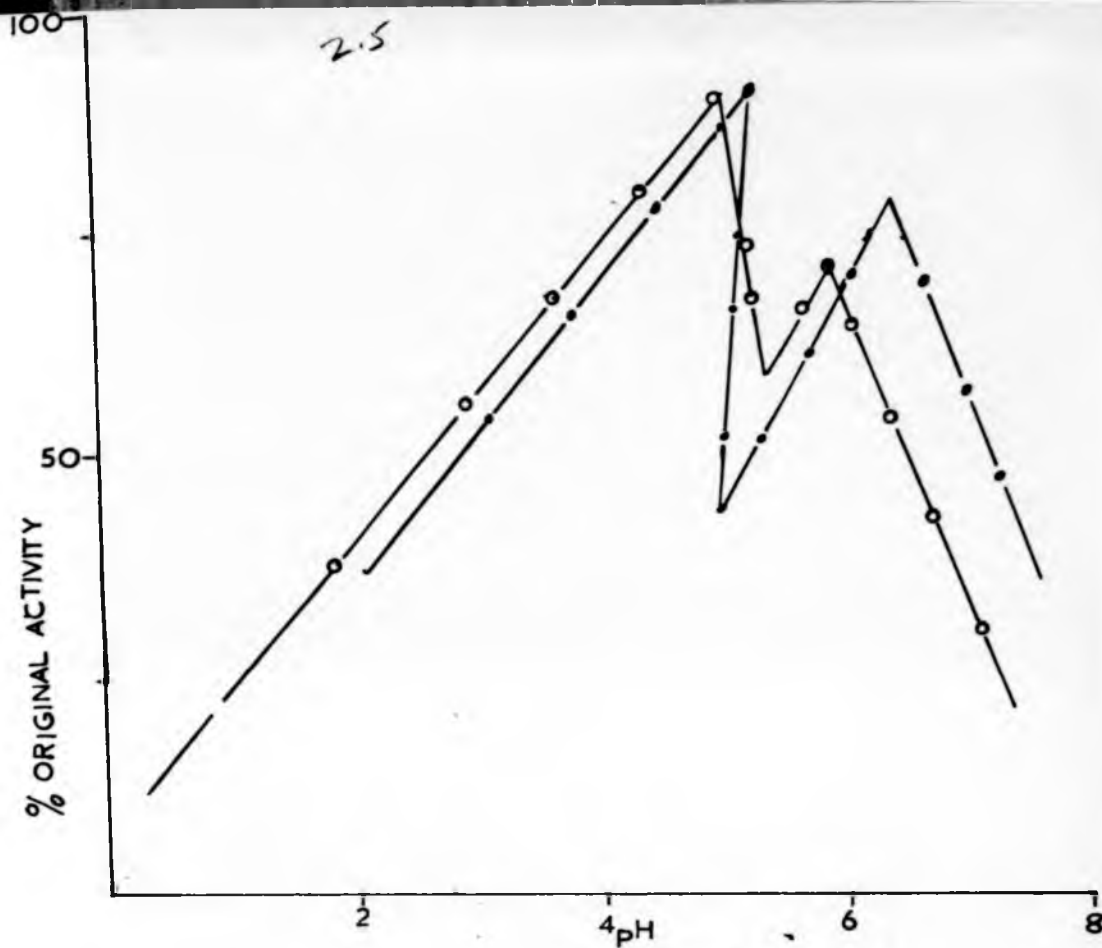
(A) Substrate specificity of phosphotransferase immobilised on Sephadex by benzoquinone. Rate of hydrolysis of 100 μ M various donors (\circ DNPP), (\times PNPP), (\bullet phenylphosphate), (\ominus p-chlorophenylphosphate) in absence of acceptor.

(B) Rate of phosphorylation of oligonucleotides (Δ) pUpUpU(3'-5'); (\bullet) pApApA(2'-5'); (\circ) pApApisopropylideneado(2'-5'); by benzoquinone Sephadex immobilised phosphotransferase.

transferase
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 donors
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 nucleotides (Δ)
 ; (o) pApAisopropyl-
 e Sephadex immobilised



- FIGURE 2.5
- (A) Effects of pH on the rate of nucleotide synthesis by immobilised phosphotransferase (●) and free phosphotransferase (○). Phosphate donor is p-nitrophenyl phosphate, acceptor nucleoside is adenosine.
- (B) Elution profile of crude phosphotransferase purified by
- (a) G-100 Sephadex
 - (b) Matrex Gel Blue A.
- (Δ) Protein concentration, in μg/ml
- (●) Transferase activity in U/ml
- (○) Phosphatase activity



- FIGURE 2.6
- (A) Effect of pH on the binding of phosphotransferase to Cibacron Blue F3GA. Experimental details in the text. (x) and (o) represent the effects of binding of the carrot and wheat shoot phosphotransferases to Cibacron \square Blue F3GA respectively.
- (B) Lineweaver Burk plot for the synthesis of (•) pApApA(2'-5') and (o) pApAisopropylidene adenosine(2'-5') by the activity of benzoquinone - Sepharose immobilised wheat shoot phosphotransferase.

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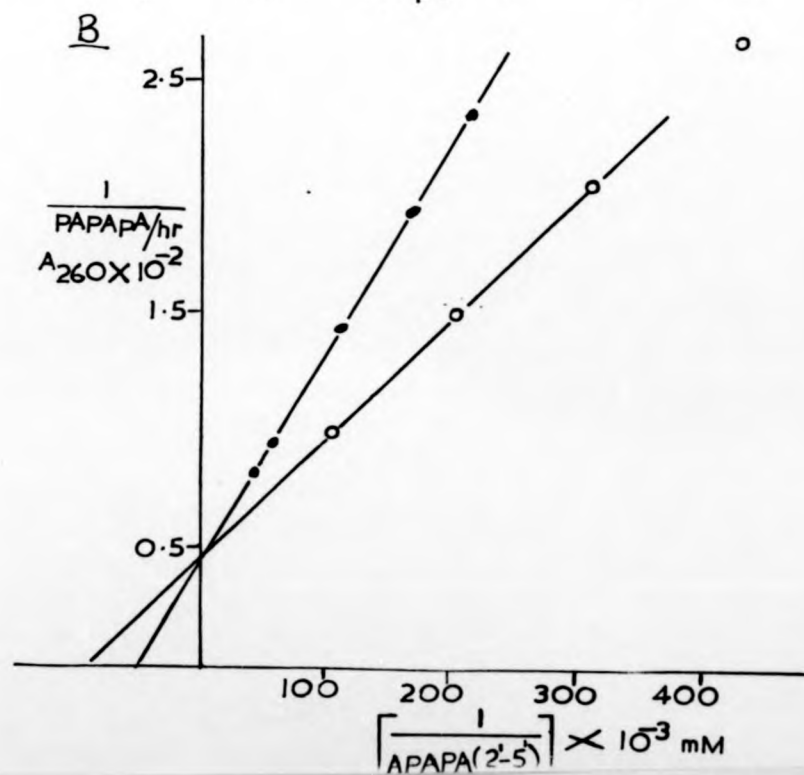
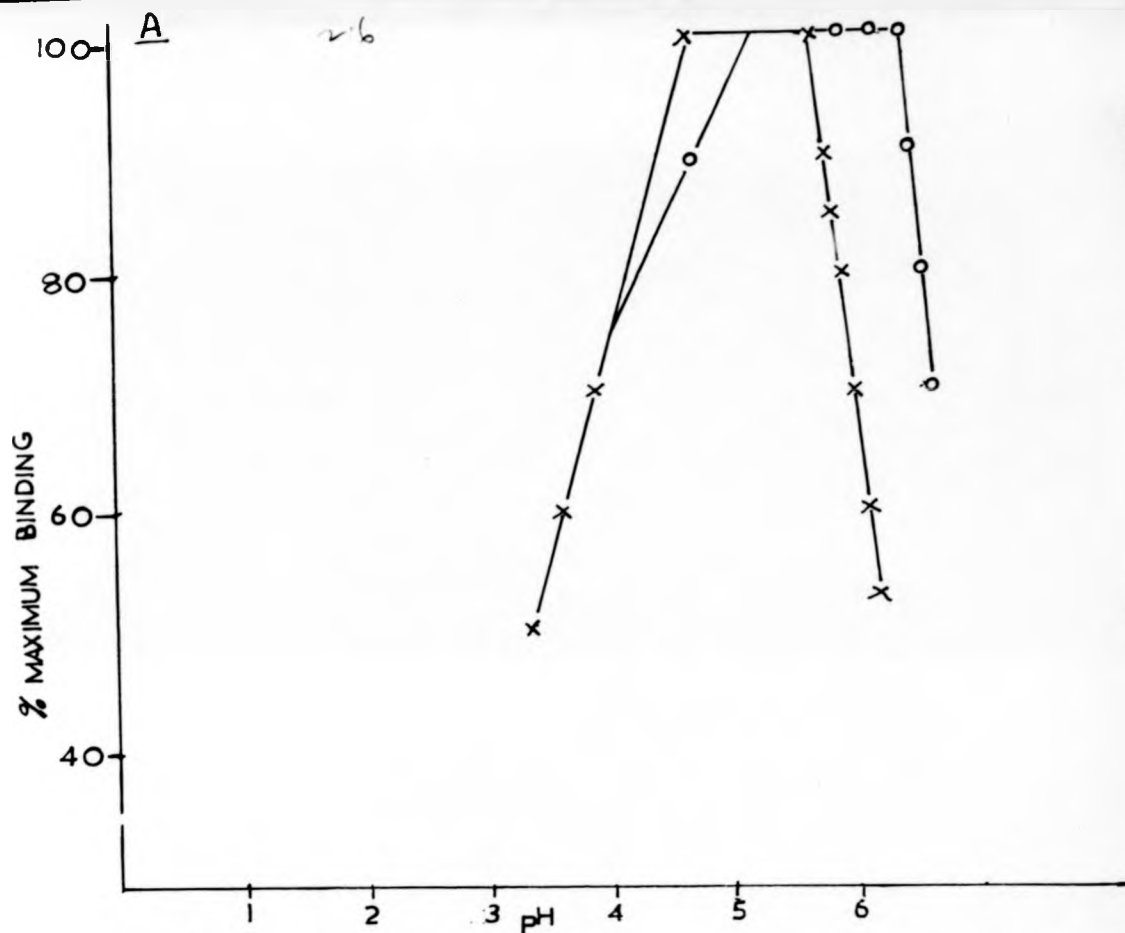


FIGURE 2.7 (A) Plot of activity versus fraction number of wheat
shoot phosphotransferase purification by
Sephadex A-25. Fraction subjected to this
purification was obtained from a previous
DEAE-Sephadex chromatography (see p.139).
Protein content mg/ml •
Transferase activity x

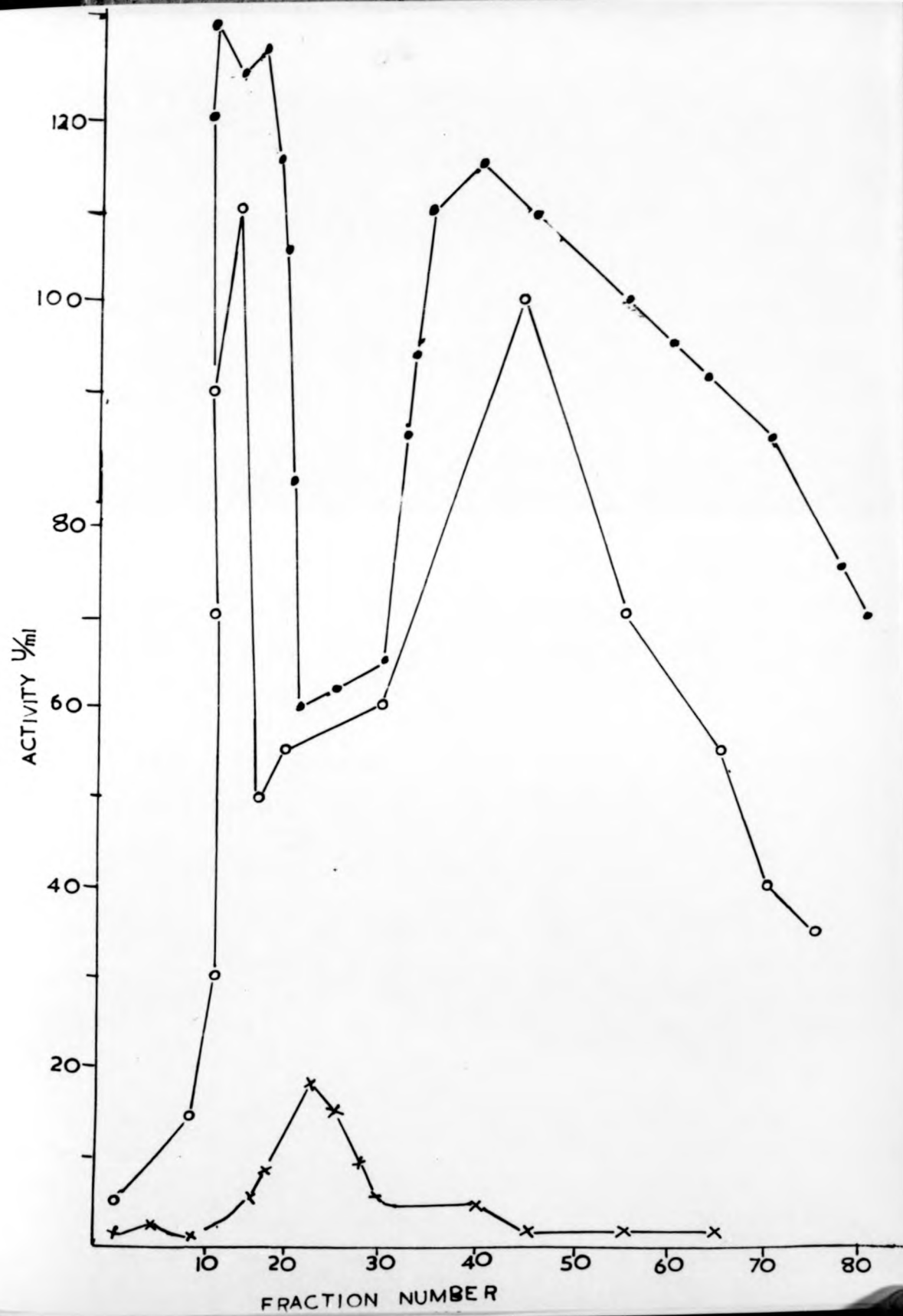


TABLE 2.1

RELATIVE YIELDS OF 5'-AMP OBTAINED USING DIFFERENT
PHOSPHATE DONORS UNDER STANDARD CONDITIONS

4-Nitrophenyl phosphate	100
2,4-Dinitrophenyl phosphate	150
Phenyl phosphate	60
4-Chlorophenyl phosphate	50
Ethyl phosphate	0

All experiments carried were out at 37°C and pH 5.0 with wheat shoot enzyme, donor (100 mM), and adenosine (25 mM) as acceptor. The yield of AMP was then determined after a fixed time interval (5 hours) when steady conditions had been established.

2.4.3 PURIFICATION OF PHOSPHOTRANSFERASE BY SUBSTRATE ELUTION FROM DEAE-SEPHADEX

A sample of the enzyme (2 ml, 30 U/ml) was dialysed against sodium acetate pH 5.0 (0.5 M) at 5° and applied to columns of DEAE-Sephadex A-25, which had been equilibrated with the same buffer. The column was washed with buffer until no more protein was eluted. Some enzyme activity was eluted, 30% of the applied units were recovered, this was in contrast to the observed 80% recovery of activity when CM-cellulose was included in the purification of the carrot enzyme (Strider, et al. 1968). However, an increase of specific activity from 0.5 to 7.9 was observed, which was consistent with the observations of the above authors.

The column was then washed with buffer containing uridine (50 mM) and fractions eluted with uridine showed 40 U/ml activity 40% of the total applied units were recovered.

2.4.4 CATALYTIC FUNCTIONS

The enzyme is considered to be a phosphotransferase with hydrolytic activity because:

- (i) Phosphate transfer is favoured over hydrolysis, see table 2.4 for k_m values.
- (ii) It transfers specifically to 5'-hydroxyl function of nucleosides and nucleotides (table 2.1).
- (iii) High yields of nucleotide are recorded (table 2.1).

Kinetic studies by Brunngraber and Chargaff (1970) are confirmed in this work

Tables 2.2-2.4, Figs. 2.4, 2.6 have shown two hydrolytic sites. One site is probably binding specifically, and the other is a transfer site, engaging the nucleoside acceptor. The kinetic constants are summarised in Table 2.4. We find, in agreement with observations of Rodgers and Chargaff (1972), that the purified enzyme from carrot always has some phosphatase activity, and, hence, there may be slow transfer of phosphate from nitrophenyl phosphate to water.

2.4.5 CHARACTERISATION OF THE TRANSFER REACTION

The simplest mechanism of a phosphotransferase appears to be based on the possibility of two active sites: one binding the phosphate donor and the other accommodating the acceptor (nucleoside or water). While this model may be true, the presence of many factors influencing the course of the transfer reaction make it difficult to accept this simple model. Some of these factors include:

- (1) Nature of the phosphate donor.
- (2) Aglycone of the nucleoside acceptor.
- (3) Sugar moiety of the nucleoside.
- (4) Donor-acceptor pair.

Of organic phosphates tested, the order of efficiencies of phosphate donors in increasing order is ethyl phosphate < 4-chlorophenyl phosphate < phenyl phosphate < 4-nitrophenyl phosphate < 2,4-dinitrophenyl phosphate under standard conditions with adenosine as the

TABLE 2.2

KINETIC CONSTANTS OF IMMOBILISED PHOSPHOTRANSFERASE

Support	Protein mg/g matrix	k_m (app.) mM	pH Optima		K cat (apparent)	
			Activity	Coupling	K cat	(free enzyme)
1. Free enzyme		2	5		100	
2. CNBr activated Sepharose	56.5	2			45	
3. Benzoquinone activated Sepharose	65	1.02	4.5	4.4	80	
Controlled Pore Glass						
(i) Adsorption	40	2.5	5-8	8	60	
(ii) $TiCl_4$ bridge	52	2.2	8	8	60	
(iii) Carboxy derivatised (pH 8) with carbodiimide coupling	27	2	8	4	40	
(iv) Carboxyl derivatised with hydroxysuccinimide coupling	18	1.92 2.3	8 7	Alkaline	56	
(v) Alkylamine derivatised with carbodiimide coupling	60	1.3	4.5	4	75	

enzyme used = enzyme preparation V

TABLE 2.3

INHIBITOR STUDIES

Substrate	Inhibitor	Type of Inhibition
p-Nitrophenyl phosphate	Dinitrophenyl phosphate	Competitive
p-Nitrophenyl phosphate	5'AMP	Competitive
p-Nitrophenyl phosphate	Adenosine	Non-competitive

TABLE 2.4

KINETIC CONSTANT FOR HYDROLASE AND TRANSFERASE FUNCTION
OF NUCLEOSIDE PHOSPHOTRANSFERASE WITH RESPECT TO
DONORS AND ACCEPTORS

Donors and Acceptors	Phosphotransferase		Phosphatase
	k_m	v_m	v_{max}
p-Nitrophenyl phosphate	3.8	0.9	2.8
Phenyl phosphate		0.7	3.0
Dinitrophenyl phosphate	3.6	1.4	2.4
Ethyl phosphate			5.0
p-Chlorophenyl			3.6
Adenosine	4.0	1.0	
Uridine	3.7	1.5	
ApA (3-5)	3.9	0.7	
ApA (2-5)	3.8	0.65	
ApApA (2-5)		0.50	
UpUpU (2-5)	4.1	1.3	

v_{max} values for the phosphatase activity of the enzyme in presence of adenosine and uridine are 2.0 and 1.9 respectively. p-nitrophenyl phosphate being the phosphate donor.
The k_i for the phosphatase activity of the enzyme in presence of uridine is 4.5.

For determination of kinetic constants of phosphotransferase activity of phosphate donors, adenosine was used as the acceptor.

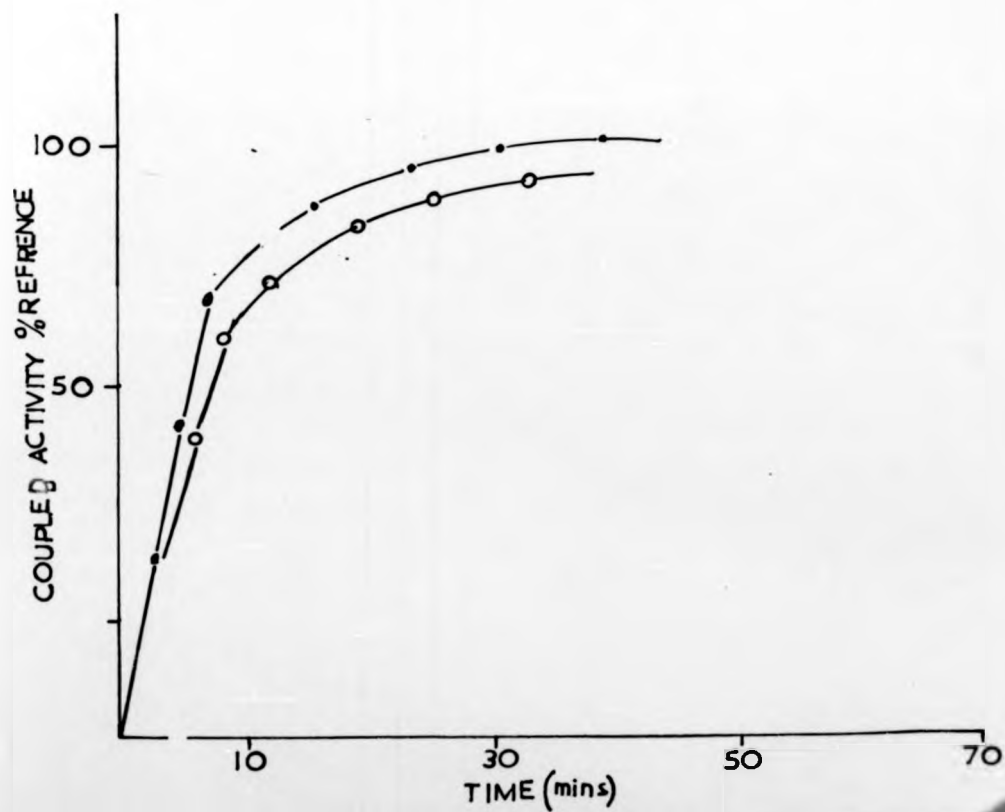
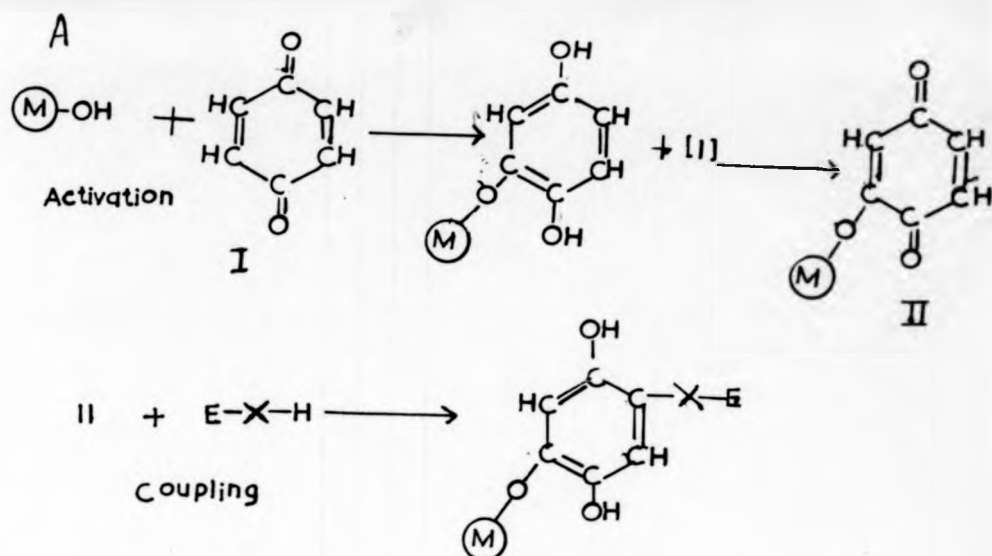
For determination of kinetic constants of phosphotransferase activities of phosphate acceptors, p-nitrophenol phosphate was used as the donor.

The values are averages of two determinations.

FIGURE 2.8

(A) Insolubilisation of enzyme by benzoquinone-activated support.

(B) Effects of time of coupling on the activity of phosphotransferase (●, 1 mg/ml) and (○, 2 mg/ml) phosphotransferase was coupled to benzoquinone-activated Sepharose at different intervals of time. The plot shows the observed activity for each preparation.



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activity of

d (o, 2 mg/ml)

enzoquinone-activated

time. The

or each

FIGURE 2.9

Derivatisation of CPG and the coupling of phosphotransferase to the derivatives. The figure shows the possible linkages through which the enzyme may be attached to the derivatives.

3. Preparation of an amide linked enzyme with alkylamine derivative and a carbodiimide. R' and R'' represent groups on the carbodiimide, e.g. cyclohexyl groups.

4. Preparation of carboxyl derivative with succinic anhydride.

5. Preparation of the pseudourea of a carboxyl derivative by reaction with carbodiimide.

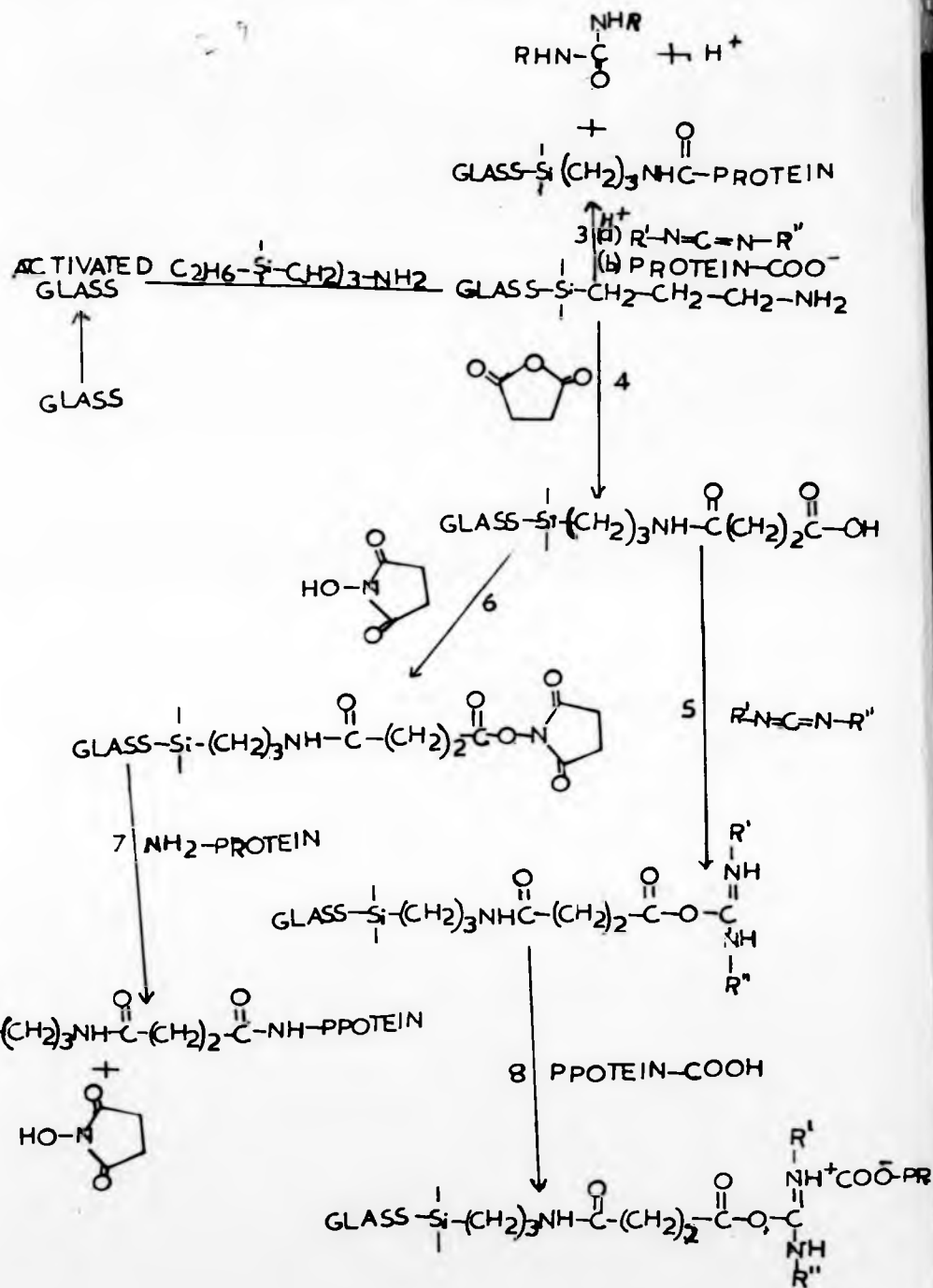
6. Preparation of N-hydrosuccinimide ester from carboxyl derivatised glass.

(a) Carbodiimide.

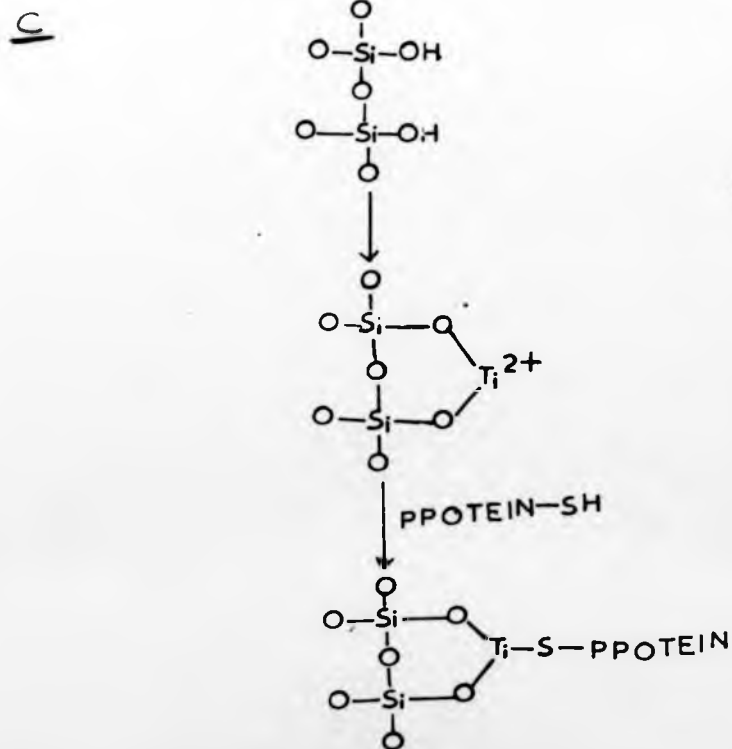
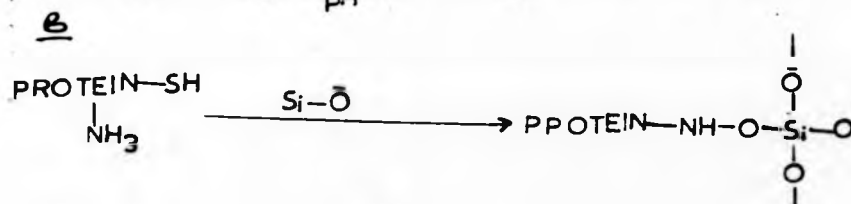
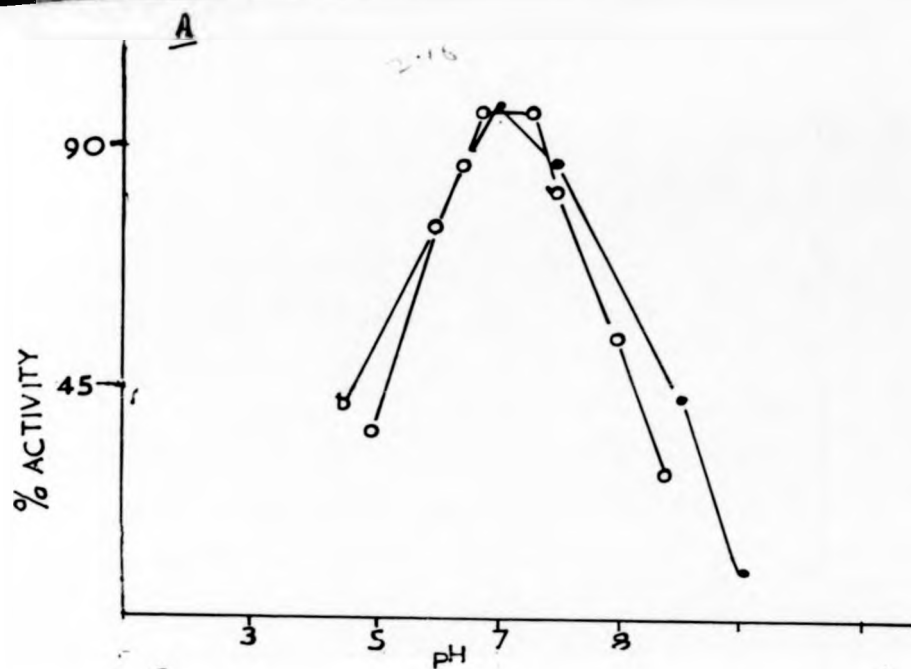
(b) N-hydroxysuccinimide.

7. Coupling of protein to the N-hydrosuccinimide ester.

8. Coupling of protein to the pseudourea of a carboxyl derivative with carbodiimide.



- FIGURE 2.10
- (A) Effects of pH on the activity of phosphotransferase (● soluble, and ○, sepharose immobilised enzyme) for the synthesis of 5'AMP.
 - (B) Linkage of enzyme to glass through adsorption.
 - (C) Linkage of enzyme to glass via TiCl_4 .



phosphotrans-
immobilised
adsorption.

4.

acceptor. Ethyl phosphate did not act as phosphate donor under the above conditions. This order probably emphasises the importance of the leaving group, and thus it may be easier to eliminate dinitrophenol from dinitrophenyl phosphate than it will be to eliminate nitrophenol from nitrophenyl phosphate. Similar effects have been observed by Brunngraber and Chargaff (1967,1970). Rodgers and Chargaff (1972) while studying the carrot enzyme, with p-nitrophenyl phosphate as donor, the acceptors can be listed in descending order of efficiency as uridine U3'p5'U3'p5'U adenosine A3'p5'A A2'p5'A A2'p5'A2'p5'A. When pA2'p5'A2'p5'A was used as a possible phosphate acceptor, no phosphate transfer occurred. Thus, in general, the pyrimidine nucleosides are better acceptors than the purine nucleosides, these agreed with earlier observations when the carrot enzyme was being studied. In addition, the nucleosides are better acceptors than oligonucleotides and the enzymatic activity is specific for synthesis of 5'-nucleotide. The relatively reduced efficiencies of oligonucleotide acceptors may indicate general presence of the secondary alcohols in the immediate vicinity of the accepting primary alcohol. As was observed for the carrot enzyme by earlier workers, with highly purified enzyme preparations, and long periods of incubation (24 hours), amounts of 2'- and 3'-isomers are produced when nucleoside was used as acceptor. However, no such isomers could be detected when oligonucleotides were used as acceptors. Thus, while there is a reduction in relative yield of phosphorylated oligonucleotide, using purified enzyme, the only product formed even on prolonged incubation is the 5'-monophosphate oligonucleotide.

The earlier workers, using carrot enzyme, indicated that phosphate transfer is limited to only one class of acceptor, nucleosides. This has been found to be true for the wheat shoot enzyme (Giziewicz and Shugar, 1975). It is therefore not strange that the oligonucleotides can accept phosphate from donors through the action of the phosphotransferase of wheat shoot. Martin et al. (1979) have similarly used the wheat shoot enzyme for the phosphorylation of trinucleosidediphosphate. However, a crude extract of the enzyme was used for the preparation, but product yield was poor due to the phosphatase activity present in the crude enzyme.

2.4.6 PHOSPHATASE ACTIVITY

The efficient phosphate donors are rapidly hydrolysed (Fig. 2.4). As was observed by Brunngraber (1978) while studying properties of the carrot enzyme, we also observed that adenosine (an acceptor for the transfer reaction) inhibits the hydrolytic activity of the enzyme. Adenosine non-competitively inhibits the hydrolysis of p-nitrophenyl phosphate, while 5'-AMP and dinitrophenyl phosphate competitively inhibit the hydrolysis of p-nitrophenyl phosphate. This is the general trend of the inhibitory action of adenosine versus the hydrolysis of the phosphate donors studied. Thus, generally, adenosine has no effect on the k_m of the various donors; it decreases the initial velocity of the hydrolysis of these donors.

This is similar to observations of Bernfeld, et al. (1957), where it was observed that in a reaction involving two substrates an inhibitor (e.g. adenosine) may be non-competitive with regard to one substrate (e.g. p-nitrophenyl phosphate) but fully competitive with regard to any other substrate.

2.4.7 EFFECT OF pH ON ACTIVITY OF PHOSPHOTRANSFERASE

The optimum pH of the purified transferase is about pH 5.0 with either adenosine or uridine as acceptor and p-nitrophenyl phosphate as the donor. Brunngraber (1978) has observed the same pH optimum for the transferase activity of carrot enzyme and that the pH optimum for release of inorganic phosphate is 5-6.5. When the enzyme is at pH 9.5, about one third of the initial transfer activity is retained (see section on immobilised enzyme).

Studies using site specific reagents by Brunngraber (1978) have indicated the presence of a histidine residue at the active site. They also observed that Na^+ , K^+ , $(\text{NH}_4)^+$, and Zn^{2+} have no effect on both activities of the enzyme, while Cu^{2+} , Mn^{2+} , and Pb^{2+} were equally inhibitory to both functions, Fe^{2+} , Fe^{3+} being more inhibitory to the transfer reaction, and we found in a trial study, Cu^{2+} enhances the phosphatase activity and NaF enhanced the transferase activity. However, whereas these authors observed a 45% enhancement of the latter activity with 0.25 M NaF using the carrot enzyme, we observed 50% enhancement with 0.2 M NaF using the wheat shoot enzyme. This may be due to the presence of relatively higher phosphatase activity in the carrot enzyme compared to the wheat shoot enzyme.

When the purified enzyme was stored at -20° for 3 weeks, no detectable loss in activity was observed. However, storage at 4° for 2 weeks led to loss of 30-40% of the transferase activity.

2.4.8 IMMOBILISED PHOSPHOTRANSFERASE

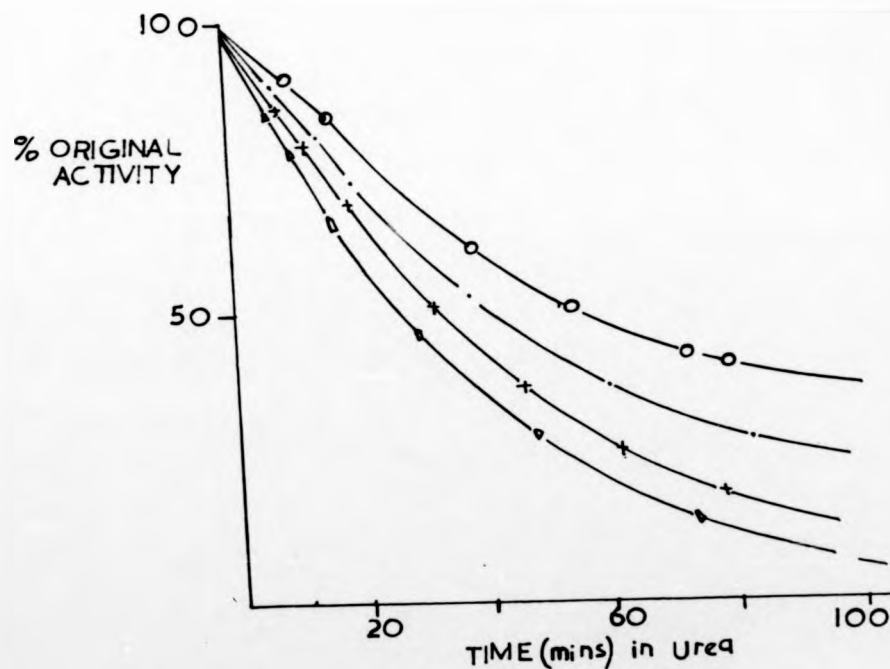
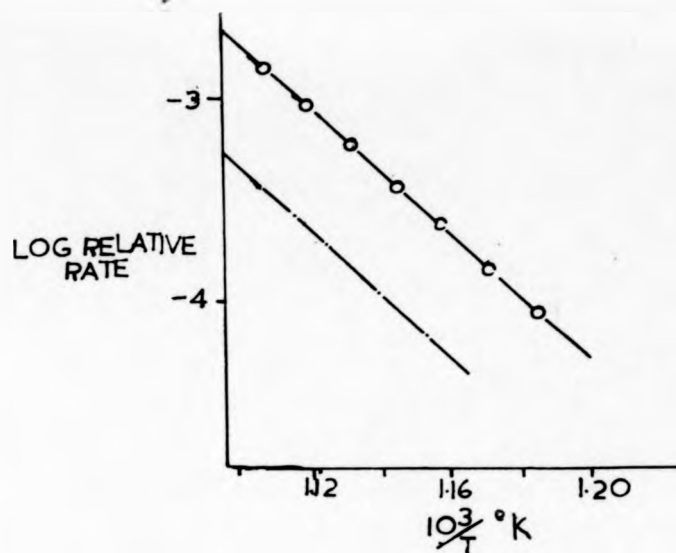
Optimum conditions for binding phosphotransferase to various supports are as follows. The conditions for the various methods of immobilisation used varied to achieve maximum activity of the bound enzyme. There are various preparations of immobilised enzyme using cyanogen bromide activated Sepharose (e.g. Hoffman, et al. 1970). The phosphotransferase was coupled to cyanogen bromide activated Sepharose, in sodium acetate pH 5.0 (0.05 M) and when necessary, the purified enzyme was dialysed against the coupling buffer. The protein content of the conjugate was often determined under varying conditions to establish optimum protein binding. The coupling of the enzyme to benzoquinone activated Sepharose was performed in sodium acetate pH 4.4 (0.1 M). Brandt (1975) obtained remarkably high amounts of albumin fixed to benzoquinone activated Sepharose at acidic pH values, this was related to group activation in connection with a conformational change which is known to take place in albumin around pH 4.0.

Coupling of the phosphotransferase to cyanogen bromide activated Sepharose at pH 8.5 produced bound enzyme whose activity was reduced compared to when the coupling was to benzoquinone activated Sepharose at pH 4.4. Since the coupling of enzymes to cyanogen

bromide Sepharose occurs predominantly through the free amino groups of the ligand protein, one of the possible reasons for reduced activity in this case may be due to the fact that the amino group interacting with the support is at or close to the active site; this interference with the active site may be responsible for the reduced activity of cyanogen bromide coupled phosphotransferase. This is analogous to the observations of Rodgers and Chargaff (1972), when it was observed that at pH 9.5 (soluble enzyme) without protecting agent, only about one third of the initial transferase activity was retained whereas hydrolase function was almost entirely lost after 24 hours. When the enzyme solution was returned to pH 5.0 a slight recovery of enzymatic activity was observed. The presence of reducing agent during the treatment at high pH provides some protection from the irreversible loss of enzyme activity. Coupling of the enzyme to benzoquinone Sepharose at pH 5.0 means that the amine in the protein is protonated, and will not react with the electrophilic support, and here the -SH group will likely react with the benzoquinone activated support. It is probable that this interaction produces an insolubilised enzyme whose conformation leaves the active site intact, thus producing the active phosphotransferase insolubilised on benzoquinone activated Sepharose (Fig. 2.8A).

For all the carrier-phosphotransferase systems, the pH optimum values for the enzymic activity were the same in both free and fixed forms. Upon adsorption on CPG-10, maximal catalytic activity of the membrane-bound enzyme was realised over a broader range of pH values. These results suggest that the microenvironmental pH of the matrices

- FIGURE 2.11
- (A) Arrhenius plot ($\log_{10} k$ against $1/T$, where T is in degrees kelvin) for the phosphorylation of adenosine by (\bullet soluble; \circ Sepharose-immobilised) phosphotransferase. For the insoluble enzyme, $E \sim 40$ kcal/mole.
- (B) Effects of incubating immobilised phosphotransferase (\circ , 1 mg/ml; \bullet , 2 mg/ml, \times , 3 mg/ml; Δ 4 mg/ml) in varying urea concentrations (see Section 2.4.9 for experimental details).



was not significantly different from the bulk pH. In a study of bonding mechanism for enzymes immobilised in inorganic support, Messing (1976) suggested that in the adsorption of enzymes to glass, charge-charge interactions (ionic, salt bridge) and hydrogen bonds were involved. Since at the acidic pH, the enzyme will have NH_3^+ as its predominant charged group, the inclusion of phosphotransferase on the glass may involve the formation of ionic bonds between the amine groups of the protein and the dissociated silanol (SiO^-) groups of the glass. The amine silicate bond may be augmented by hydrogen bonds during interaction of protein to glass (Fig.2.14). Inorganic bridge formation between the enzyme and glass via titanate chloride can be explained as follows: since titanate ion, unlike glass (silica), readily undergoes redox reactions, the linkage of the enzyme could be attributed to changes in oxidation state of the titanate chloride due to electron transition that occurs during the reaction of silanol (SiO^-) groups of glass with titanate chloride when the latter interfaces with the sulphhydryl group of the protein and immobilised enzyme results. The sulphhydryl group of phosphotransferase is one of the possible functional groups on the surface of the protein capable of readily undergoing redox reactions. This appears to indicate that binding between titanate chloride and phosphotransferase may at least be partially covalent in nature. This may be in addition to simple ion-ion interactions and hydrogen bonding.

Fig.2.9 shows the derivatisation of CPG and coupling of the phosphotransferase to these derivatives, the figure shows the possible mode of linkage of the enzyme with the various derivatives

As mentioned earlier, a plateau-like pH optimum was obtained on the adsorption of the phosphotransferase to CPG-10, and similar slight variations in optimum were observed on immobilisation of the enzyme. Chemical composition of Sepharose and the microstructure of the CPG lend themselves to influences on environmental effects. For example, by blocking amino or carboxyl groups or by introducing additional charged groups on the carriers, the surface charge could be altered; this in turn could modify the local pH. A highly negatively charged carrier would attract hydrogen ions, maintain a lower environmental pH than the bulk of the solution, and thus stabilise the enzyme in a highly alkaline solution (e.g. phosphotransferase coupled to CPG via titanate chloride, pH optimum 8.0) and vice-versa. The latter could be the case in the plateau-like optimum obtained on adsorption of phosphotransferase to CPG-10 (Beck and Rase, 1973).

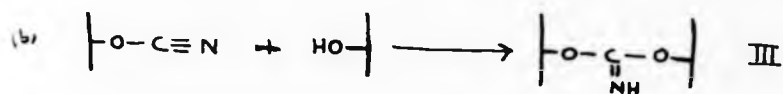
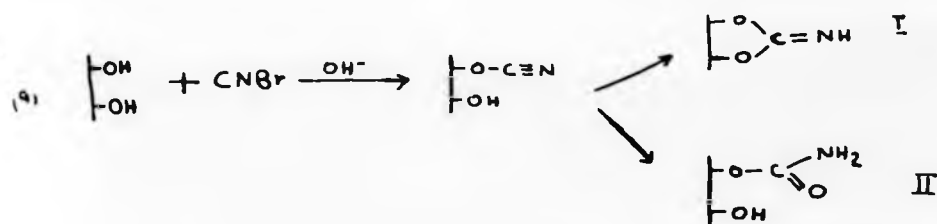
In most other cases (Table 2.2) the pH optimum of the phosphotransferase immobilised was almost similar to that of the soluble enzyme.

The rate of formation of oligonucleotide 5'-phosphate synthesised by the immobilised enzyme increases with temperature over the range 30-40°C (Fig. 2.110). Shows the Arrhenius plot obtained for immobilised and soluble phosphotransferase.

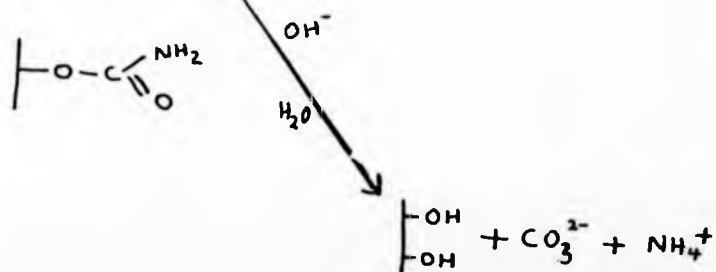
It was observed that the p-benzoquinone activated Sepharose binds more protein than cyanogen bromide-activated Sepharose. Phosphotransferase from wheat shoot used here has a molecular weight 45,000, and the highly porous nature of the Sepharose matrix allows the easy diffusion of the protein in and out of the gel during the preparation of the immobilised derivative. Thus, immobilisation of the enzyme could occur either in the interior or exterior of the

support. The same support (Sephadex) being used in the case of benzoquinone and cyanogen bromide activations means that the differences in protein content of the immobilised enzyme in the two methods is not due to internal immobilisation of the enzyme in the interior of the support. The reason could be attributed to the higher reactivity of quinones (Fig. 2.8A) than the cyanogen bromide, and the former will attack the hydroxylic polymer more readily than cyanogen bromide. In addition, when cyanogen bromide reacts with the support, in addition to the reactive imidocarbonate produced, unreactive carbamates are produced too. The reactive imidocarbonate may be hydrolysed to carbonate, and further back to the original matrix and further complication includes the undesirable hydrolysis of the reagent itself with the production of bromide, cyanide and cyanate (Fig. 2.12). The net effect of the above factors is to make benzoquinone-Sephadex a better electrophile than the cyanogen bromide-Sephadex, and thus the former will be more reactive towards the nucleophiles present in the enzyme. The reactivity towards nucleophilic groups in protein follows the order $\text{SH} > \text{NH} > \text{OH}$. Nucleophilic attack by aliphatic hydroxyl groups takes place at a strongly alkaline $\text{pH} \sim 11$ while amino groups react at a lower pH, and SH groups couple more readily at lower pH. It can thus be expected that readily accessible SH groups on the enzyme surface will couple to benzoquinone Sephadex, and the relatively high enzyme activity of this derivative may be either due to the fact that the SH group reacting with the support is not at the active site of the enzyme, and thus geometry of immobilisation does not interfere with the active site of the enzyme, or that if the SH groups reacting with the polymer are at the active site, the precise three-dimensional

FIGURE 2.12 Type of reactions during the activation of polysaccharide matrix with cyanogen bromide.



(c)



I = imido carbonate

II = Unreactive Carbamate

III = hydrolysis of imidocarbonate(I) to carbonate IV

- (a) Reaction of Cyanogen bromide with Sepharose
 (b) Possible side reactions of the cyanates produced in (a).
 (c) Hydrolysis of the reactive imidocarbonates to produce carbonate (IV) or hydrolysis of the imidocarbonate to the original matrix.

TABLE 2.5

(a) DEACTIVATION RATE CONSTANTS OF IMMOBILISED AND SOLUBLE ENZYME AT 35° ($\text{min}^{-1} \times 10^3$) AS A FUNCTION OF pH

pH	Soluble	Immobilised
3	10	9
4	3	21
5	4	2
6	9	15
7	45	50

(b) DEACTIVATION RATE CONSTANT $\text{min}^{-1} \times 10^3$ (AT pH 4.5) AS A FUNCTION OF TEMPERATURE

Temperature	Soluble	Immobilised
30	1.5	1.0
40	4.5	2.5
50	6.0	4.0
60	15.0	8.0
70	40	16

In (a) the soluble and immobilised enzymes were treated with buffers at various pH's and incubated at 35°, in (b) the enzymes were incubated in pH 4.5 at various temperatures for 5 mins.

The values are averages of two determinations.

arrangement of charges, microenvironment and geometry of the reaction may have allowed the active site to survive the coupling procedure. The lower activity obtained in cyanogen bromide Sepharose immobilised enzyme, may be due to either active site modification, non-accessibility of the active site to incoming substrate, or some damage to the active site at the necessarily high alkaline pH used to immobilise the enzyme to cyanogen bromide support.

While the enzyme coupled to cyanogen bromide activated Sepharose showed little variation in activity with protein content, enzyme coupled to benzoquinone-activated Sepharose showed a more prominent increase in activity as the protein content decreases. This is probably due to steric hindrance. It is therefore efficient and economical to immobilise a small quantity of enzyme on the support rather than trying to obtain the highest loading, since at a point of high protein loading, reduction in activity-ratio of bound to free enzyme was observed. Axen and Ernback (1971), observed that specific activity of enzyme after immobilisation tends to go down with increasing protein contents in the conjugates.

2.4.9 STABILITY OF THE IMMOBILISED PHOSPHOTRANSFERASE TO THERMAL DENATURATION AND INACTIVATION BY UREA

The thermal stability of the enzyme was studied in both the free state and after immobilisation. Fractions of the enzyme were incubated at room temperature, rapidly cooled and then assayed under standard

conditions for phosphorylation activity. The results obtained with the phosphotransferase are shown in Table 2.5b. Immobilisation on benzoquinone-Sepharose resulted in a marked increase in thermal stability.

The stability of the enzyme to denaturation by urea was determined by incubating aliquots of the immobilised enzyme in urea (1.0 M) at 37°C. At a given time interval, aliquots were transferred to a water jacketed vessel and assayed for activity at the same urea concentration. Results are shown in Fig. 2.11A. The derivative with lower protein content showed greater stability, retaining > 70% of its activity after 30 min. incubation, while the higher protein content derivative retained > 50% of its original activity after 30 min. This may reflect changes in conformational flexibility which accompany immobilisation of the enzyme.

The temperature stability of enzyme bound to the support was higher than that of the soluble enzyme (Table 2.5). After heating for 5 min. at various temperatures the immobilised enzyme showed lower rate of deactivation compared to the soluble enzyme under similar conditions; this may be due to the fact that enzymes in solution are subjected to intramolecular interaction, and thus heat will enhance protein aggregation, which in turn may decrease soluble enzyme activity upon heating. Burgess et al. (1975) have observed that carboxypeptidase A bound to cyanogen bromide Sephadex, retained a considerable amount of its activity at temperatures up to 70°C, whereas the soluble enzyme lost its activity.

Studies of temperature, storage and urea stabilities of the immobilised phosphotransferase thus indicated immobilisation

has induced new properties into the bound enzyme, and that these changes of the molecular properties of the enzyme upon immobilisation have led to new operational aspects (in terms of improved stabilities to variety of conditions) and in turn enhanced the applications and uses of the immobilised phosphotransferase.

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CHAPTER THREE

3. PHOTOAFFINITY LABELLING

3.1 PHOTOAFFINITY LABELLING OF BIOLOGICAL SYSTEMS

The molecular mechanisms resulting in observed biochemical processes are major targets for chemical and biochemical research. These molecular mechanisms often involve the interactions of biological processes, the identification and the structural characterisation of the component complex systems. Up to the present, groups of enzymes have been isolated, their biological substrates identified; these have contributed to the understanding of their mechanisms of action. Little is known about their detailed mechanism and geometries of active site.

One approach to obtaining this information involves the use of the method of affinity labelling, whereby specific reagents for chemical modification of active site residues are used (Singer, 1967). The use of affinity labelling is based upon the fact that the binding of most biological ligands to their specific receptor sites involves a number of favourable interactions that together make up the ligand-receptor recognition process. The total bonding energy is usually high enough to allow some latitude in modifying the structure of the natural ligand without excessively sacrificing either the selectivity or the strength of the overall binding interaction. This often allows the incorporation of a chemically labile group into the ligand with retention of most of the structural features that are required for recognition by the receptor macromolecule. The modified ligand can then become an

affinity label, and may lead to selective modification of the binding site if appropriate chemically reactive functionality is present at the ligand binding site. This technique has been useful in the selective labelling of receptor sites in a large variety of biological systems (Schoellmann and Shaw, 1962). The technique suffers several drawbacks including the possibility of disturbance of the recognition process due to the incorporation of appropriately reactive labile groups, and secondly, there is limitation to the number of chemically reactive groups that can be incorporated into the ligand, due to the fact that the use of a water labile ligand may lead to its hydrolytic destruction before it can reach the binding site. This is especially important since a majority of chemical affinity labels are electrophilic species. Their successful deployment depends on the successful competition between them, at the binding site, and the 55 M water as solvent in which the labelling reaction is carried out. Furthermore, for studies of cellular processes, classical chemical reagents can lead to reactions with reactive groups outside the cell where no incorporation of reagent into the active site occurs. These major problems can be circumvented by the use of photogenerated reagents (Knowles, 1972, and Creed, 1974). The method involves the modification of the natural ligand by the incorporation of a chemically inert but photochemically labile group, so that when the system is irradiated, the photolabile group is converted to a species of very high chemical reactivity. Ideally, the reactive reagent so generated at the ligand binding site, reacts indiscriminately with whatever chemical groups it finds there. The high indiscriminate reactivity of such reagents can provide

valuable information on the constitution of binding and of catalytic sites of isolated macromolecules in addition to tagging and therefore identifying different kinds of macromolecular receptors. This technique is promising and will give a more ideal labelling reagent than previously used techniques of chemical modification of active site residues by suicide-inhibition (whereby due to catalytic activity of an enzyme, reactive molecule is generated at the active site from an 'inert' precursor used. The reactive molecule, mostly an allenic moiety, functions as a chemical trap for a nucleophilic residue on the enzyme (Miesowicz and Bloch, 1975)).

The use of photogenerated reagents (photoaffinity labelling) to study biological macromolecules was introduced for the photolysis of diazoacetylchymotrypsin by Singer, et al. (1960). Since then, the technique has evolved into a major method for studying molecular interactions in biological systems (Bayley and Knowles, 1977). Some of the criteria for an effective photoaffinity reagent were given by Bayley and Knowles (1977). They include the fact that:

- (1) The label should be chemically inert in the aqueous solution used in biological systems.
- (2) The smooth photolytic conversion to reactive species should be accomplished at wavelengths clear of the absorption of the receptor system.
- (3) Rearrangement of the reactive species should not occur as this is likely to lower the reactivity and hence non-specificity of insertion, drastically.
- (4) The label must be easily synthesised using stable reagents.
- (5) The rate of reaction of the species generated should be

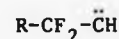
extremely high so that diffusion of the reagent out of the ligand-binding site cannot occur before covalent insertion takes place.

Commonly used precursors are compounds containing the diazo or azido groups which, upon photolysis, can be expected to generate carbenes and nitrenes respectively, both of which are highly reactive intermediates which insert into C-H, O-H and N-H bonds.

Carbenes were first suggested as possible photoaffinity labels (Jones and Moss, 1973 and 1975), but they react rapidly with a variety of chemical functions: by coordination to nucleophilic centres (to give carbanions), by addition to multiple bonds (including aromatic systems), by insertion into single bonds (including carbon-hydrogen bonds), and by hydrogen abstraction (to give two free radicals that may then couple). However, if there is a hydrogen atom on the carbon atom adjacent to the carbene centre, hydrogen migration readily occurs, and results in unreactive olefine:



Hence, the adjacent atom must bear no hydrogen:



Also, the α -keto carbene species, although attractive for a number of other reasons, may undergo the intramolecular Wolff rearrangement resulting in a ketene:



This rearrangement may occur on photolysis without the intermediacy of the carbene (Roth and Manion, 1976). These problems, including difficulties in synthesis, short wave lengths necessary for irradiation, and high reactivity, point to the fact that the carbenes are not ideal photoaffinity labels. Since the original experiments conducted by Singh, et al. (1962) with diazoacetyl derivatives of chymotrypsin, there have been numerous reports of the use of photoaffinity labels. The majority, however, have used azides to generate nitrenes.

Apart from the recently used aryldiazirines, a member of the carbene group, (Smith and Knowles, 1975), the nitrene precursors especially arylnitrenes, have been the most used in photoaffinity labelling experiments. The arylnitrenes can satisfy most of the criteria listed above. The only disadvantage of the nitrenes involves their relatively longer half-life, which reduces their rate of reactivity and hence increases the chances of non-active site interaction or non-specific-labelling. This is so because the rates of reaction of the species being generated by nitrene precursors are relatively low, and diffusion of the reagent out of the ligand-binding site can occur before covalent insertion takes place. This, therefore, makes the arylnitrenes less reactive compared to corresponding carbenes, and thus insertion reactions of the nitrenes are more discriminating than the insertion reactions of carbenes (Reiser, et al. 1968). Ruoho et al. (1973) observed that the photolysis of acetylcholinesterase of erythrocyte membranes (AChE), with 4-azido-2-nitrobenzyl trimethylammonium ion

was accompanied by a significant amount of non-specific labelling. The latter was reduced ten-fold upon photolysis in the presence of p-aminobenzoate, a scavenger that is believed to react with the photogenerated reagent present in the solution rather than at the binding site. Importantly, no photoinduced inactivation of AchE was observed in the presence of added scavenger. Thus, inactivation did not occur by reaction of the photogenerated nitrene before it diffused out of the active site, but the reactive species had lifetime sufficient to diffuse out of the active site several times over before reacting with it. Such labelling is called pseudo-affinity and it is similar to conventional affinity labelling. The use of scavenger, which acts as a 'mop' is therefore an important method which will clearly differentiate between photoaffinity and pseudo-affinity labelling. Scavenger experiments must therefore always be included during photoaffinity studies.

Apart from the use of scavengers, because of the potential of the photoaffinity reagents in terms of random labelling, it has become necessary to control photoaffinity labelling experiments very carefully, for a genuine site-specific and u.v.-dependent incorporation. It must be shown that no incorporation or loss of activity occurs in the absence of u.v. or when using pre-irradiated label. Photolytic treatment must not damage the macromolecular receptor, and any protection afforded by substrate analogues must not be due to their self-absorption of light, which if it happens, may preclude photolytic destruction of the label itself.

Since prolonged photolysis of a ligand-protected system will still

yield a time-dependent inactivation of the receptor, due to reversibility of the physical system being dealt with, therefore at any time there will be a constant diffusion of ligand into and out of the active site, and as long as any label remains unphotolysed, inactivation can continue slowly. The perturbation caused by the reactive group, or its precursor, to the molecular interaction being examined, must be kept to a minimum. Finally, if precise information about molecular interaction is desired, it is imperative to establish that the probe not only binds to the same site but also that it binds in a mode identical to that of the true substrate. If, however, the experiment is designed only to identify and tag the binding sites or to identify specific receptors in a complex milieu, demonstration of competitive binding and true photoaffinity labelling will suffice even if the precise mode of binding is not known (Chowdhry and Westheimer, 1979),

The presence in a natural ligand of a functional group, such as an α , β -unsaturated system, might provide in some instances an advantage over reagents that incorporate synthetic photolabile groups, especially if the photochemical behaviour is well understood. Thus, Martyr and Benisek (1973), have used photo excited α , β -unsaturated keto steroids to inactivate Δ^5 -keto steroid isomerase, and recently, amino acid analysis and sequence analysis have shown that the photoinactivation is accompanied by the transformation of aspartic acid 38, to an alanine residue. No mechanism for this novel photochemical reductive decarboxylation has been proposed (Ogez, et al. 1977).

Several investigations have shown that cyclic nucleotides undergo photoincorporation into different receptors. Thus, Guthrow, et al. (1973), observed incorporation of N⁶-(ethyl-2-diazomalonyl)-adenosine 3',5'-cyclic monophosphate into intact ghosts from human erythrocytes on photolysis at 253.7 nm. Kallos (1977) has used the same derivative to photolabel a cytoplasmic receptor. Antonoff and Ferguson (1974) have used both photolabile derivatives of cyclic AMP and cyclic GMP in photoaffinity studies of cyclic nucleotide receptors in extracts from testis, adrenal cortex and in messenger-like ribonucleoprotein-like particles.

Thus, when all criteria have been satisfied, the technique of photoaffinity labelling appears to offer peculiar advantages over other techniques in studying particular systems. The only other problem involves the fact that the number of products generated in the binding site may be several, arising from different modes of insertion into one amino acid, and the probability that more than one amino acid will be labelled due to the rotational motion of the label prior to reaction. The analysis of, for example, an enzyme active site so labelled may be fairly complicated, given that modified peptides might be difficult to detect in normal fingerprinting systems, and amino acids may be modified in several different ways making investigation all the more difficult (for example, see the reference of W. F. Benisek above in the case of Δ^5 -keto steroid isomerase). However, it appears to hold much more promise in the field of identifying ligand binding sites within macromolecules and more complex arrays as well as identifying sites of action of both

small and large ligands, e.g. hormones or polynucleotides in multicomponent systems such as organelles or in cellular tissues. It is also of particular usefulness for subunit identification (Cartwright and Hutchinson, 1980).

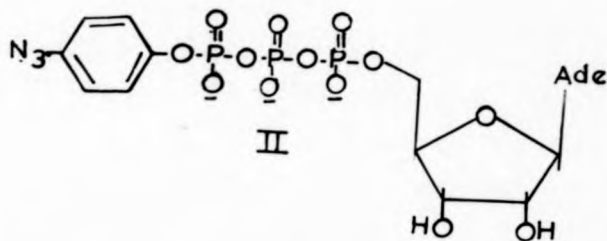
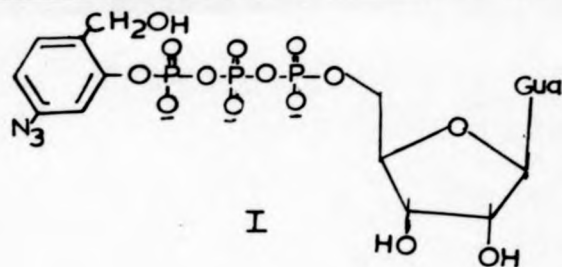
However, success has been obtained by many groups studying photochemical cross-linkage of protein to nucleic acids, generally utilising the photoreactivity of nucleic acid.

3.2 PHOTOAFFINITY LABELLING USING NUCLEOTIDES

Taking the whole studies of photoaffinity labelling up to date, widespread use of affinity labelling to investigate small ligand-protein interactions in vitro, has been accomplished. However, relatively little research has been carried out on corresponding nucleic acid-protein interactions, obviously due to difficulties in product analysis mentioned above, and possibly due to synthetic problems of introducing suitably reactive groups into the nucleic acid. There are exceptions and these include tRNA, which has been modified both at the 3'-end and at the 4-thiouridine base. The introduction of an electrophile into nucleic acid to form a potentially large number of reactive sites may lead to difficulties in obtaining true equilibration with a given protein, owing to rapid non-specific reactions. But, the promising results obtained by the use of photoaffinity labelling provide grounds for optimising the development of suitable model systems in the area of nucleic acid interactions with their receptors which would be of great importance. Such interactions that involve nucleotides as

- FIGURE 3.1 (A) Structure of two nucleotidic, azide-containing photoaffinity labels used successfully for labelling of macromolecules. (See text).
- (B) (i) Photolytic decomposition of azides and diazo compounds.
- (ii) General reactions of nitrenes.

A



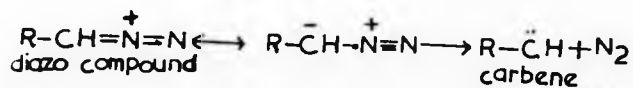
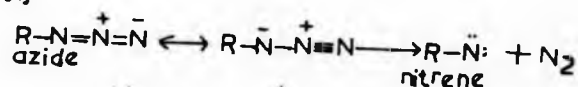
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B

[i]



[ii]

Recombination



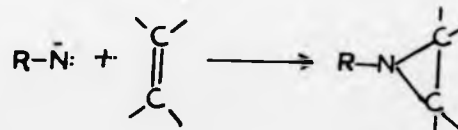
Insertion



Abstraction



Addition



essential elements include, enzymic systems where nucleotides can act as substrate, allosteric effector or cofactor, cyclic nucleotide receptors in membranes or in intracellular locations, transport mechanisms, involvement in both the nucleic acid transcription and replication, and protein synthetic machinery.

A variety of modifications has been made to purine ribonucleotides to provide photoaffinity labels suitable for these different applications. Attachment through the phosphate moiety has been used to identify a GTP binding protein in E.coli ribosomes by irradiation of P^1 -(3-azido-6-hydroxymethylphenyl)- P^3 -(5'-guanyl)triphosphate I (Fig. 3.1A). P^1 -(4-Azido)- P^3 -(5'-adenyl)triphosphate II (Fig. 3.1A) has also proved useful in studies of aminoacylation reactions of aminoacyl tRNA synthetases (Akherdyan, et al. 1977). Ribose modifications at the 2'-(3')-position by arylazido derivatives have been employed to label mitochondrial ATPase using ADP (Lunardi, et al. 1977). Modification of the base has, however, been largely confined to introduction of the azido group at the 8-position of the purine ring.

One of the earlier uses of z^8Ado nucleotides in photoaffinity labelling was by Haley and Hoffman (1974) who investigated the interaction of z^8ATP with ATPases of erythrocyte ghosts. The ATPases were specifically and irreversibly labelled by the analogue. The labelling could be abolished in the presence of the normal substance. It was felt that such z^8Ado nucleotides were highly effective, fulfilling the criteria required for photoaffinity labels and likely to prove a powerful tool in probing nucleotide binding sites. Following this suggestion, numbers of successful reports of the use of z^8Ado nucleotides in a variety of systems

have been reported. This includes cAMP sites on cellular membranes at intracellular locations (Skare, et al. 1977) and subunit localisation on protein kinases (Pomerantz et al. 1975).

Photoaffinity labelling has been used to detect binding interactions in complex systems and subunit topography (Pellegrini and Cantor, 1977). However, the use of photoaffinity technique for detailed characterisation of the binding site in terms of location of those amino acids important in contact, binding or catalytic functions, has not been a complete success. While detailed investigations on trypsin and chymotrypsin after photolysis of the diazoacyl intermediates have revealed labelling of a cysteine residue in both cases (Hexter and Westheimer, 1971), Bridges and Knowles (1974), investigating the same system found, on analysis, a number of radioactive peptides arising from photolysis of 4-azido (^{14}C)cinnamoyl α -chymotrypsin and several peptides were found in a similar study on subtilisin (Stefanovsky and Westheimer, 1973). The photochemistry of insertion of carbenes or nitrenes into amino acid residues is very little understood, so that identification of altered amino acids presents difficulties and is likely to be attempted by difference in amino acid analysis rather than by positive identification. The ability to measure the degree of reversible interaction at the binding site by obtaining k_A , k_I or k_M values in the absence of activating light is one of the advantages of photoactivated probes. After the kinetic values have been obtained, they may be used to optimally design the photoincorporation experiments.

The z^8 -AMP has been used in a number of reports for photoaffinity label, especially in the form of z^8 -cAMP (Haley, 1976).

The reagent is converted to the nitrene form by photolysis with ultraviolet in the range 240-320 nm (Fig. 3.18). As mentioned earlier nitrenes are very reactive and will form covalent bonds with amino acid R groups. The possible reactions of azides via photogenerated nitrenes have been previously discussed. The bond formed when z^8 -AMP is photoincorporated into membrane proteins is stable to acid treatment, at least at 52° and probably higher and to boiling at pH 9.0. Nothing has been found that will remove photoincorporated, covalently bound label without also destroying the primary protein chains.

z^8 -AMP can have a strong affinity for AMP binding sites including systems such as AMP binding proteins present in cells and cell membranes, and purified kinases (Haley, 1976; Pomerantz, *et al.* 1975; and Haley and Hoffman, 1974). In this work we have found that it has a strong affinity for staphylococcal nuclease an enzyme for which AMP is a competitive inhibitor. The interaction of z^8 -AMP with the enzyme staphylococcal nuclease, was investigated as a model system as the active site had a known geometry. This was initiated on the premise that by defining the reaction characteristics of the photo probe with a relatively simple target, optimum conditions could be achieved more easily in experiments with the following.

- (i) The relatively more complex systems of antibodies and ribosomal systems.
- (ii) Tissue culture conditions, e.g. interferon receptors and other complex receptors and transport systems.

The choice of staphylococcal nuclease as a model was encouraged by the fact that it is a relatively simpler system, as said before, and the enzyme has been well studied and characterised,

and importantly, the enzyme lacks SH and S-S bonds (Anfinsen et al., 1974). Since these bonds can be affected by u.v. radiation, their absence in the native enzyme provides an ideal enzyme system which can be investigated with a photolabile reagent.

3.3 STAPHYLOCOCCAL NUCLEASE

3.3.1 SUBSTRATE SPECIFICITY AND CATALYTIC MECHANISM

Polynucleotide Substrate

The enzyme hydrolyses DNA and RNA to produce 3'-phosphomononucleotides. It has a molecular weight of 16,800, 149 amino acid residues and lacks SH and S-S bonds. It is inhibited by 5'-ribonucleotides. Protection from inhibition was achieved in the presence of 3'-nucleotides and divalent cations (Cuatrecasas, et al. 1967). The specificity attributed to the enzyme makes it a useful tool in nucleic acid and oligonucleotide studies as it is less specific to constituent bases than it is to substrate conformations (Taniuchi and Anfinsen, 1966).

The mechanism of catalysis is relatively complicated. The cleavage proceeds in two stages, first an endonucleolytic attack on the nucleic acid at groups -xpA- and -xpT- to release oligonucleotides; the newly formed oligonucleotides terminating in -xp are rapidly digested exonucleolytically in regions with a high density of Ap and Tp residues. Both endo- and exonucleolytic cleavage occur simultaneously, the function of the former is to loosen the secondary structure to permit more rapid attack at newly formed ends (primary exo). Generally, the endo- and

exonucleolytic digestions prefer the -xpA- and -xpT- and Ap,Tp terminating residues respectively (Cuatrecasas and Wilchek, 1977; Taniuchi and Anfinsen, 1966; Dirksen and Dekker (1960). showed in their studies that mononucleotides are preferentially released from 3'-phosphate ends in the exonucleolytic step of the enzyme action. However, more work on oligonucleotides will ascertain whether the exonucleolytic action of the micrococcal enzyme occurs exclusively at 3'-phosphate ends or not.

Rate of hydrolysis can be assayed by sensitive spectrophotometric estimation of hyperchromicity occurring on hydrolysis of substrate. Staphylococcal nuclease has 7-fold affinity more for denatured DNA than for RNA (Taniuchi and Anfinsen, 1966).

The pH optimum is 9.0-10.0 and at higher pH less Ca^{2+} is needed. Enzymic activity depends on Ca^{2+} and the precise role of Ca^{2+} is still being studied, but it is known not to be involved in producing conformational changes in the substrate since Ca^{2+} is needed for binding of nucleotide inhibitor to enzyme active site; the binding to the substrate is also non-specific (Cuatrecasas and Wilchek, 1977). If Ca^{2+} binds first to the substrate, the binding is dependent on the interaction of substrate with enzyme, and this in turn depends on the interaction of Ca^{2+} with substrate.

3.3.2 SYNTHETIC SUBSTRATES AND INHIBITORS

Various studies have shown that the basic nucleotide substrate need not be a polynucleotide chain. Compounds of general class

FIGURE 3.2 Reagents used for affinity Labelling of Staphylococcal Nuclease .

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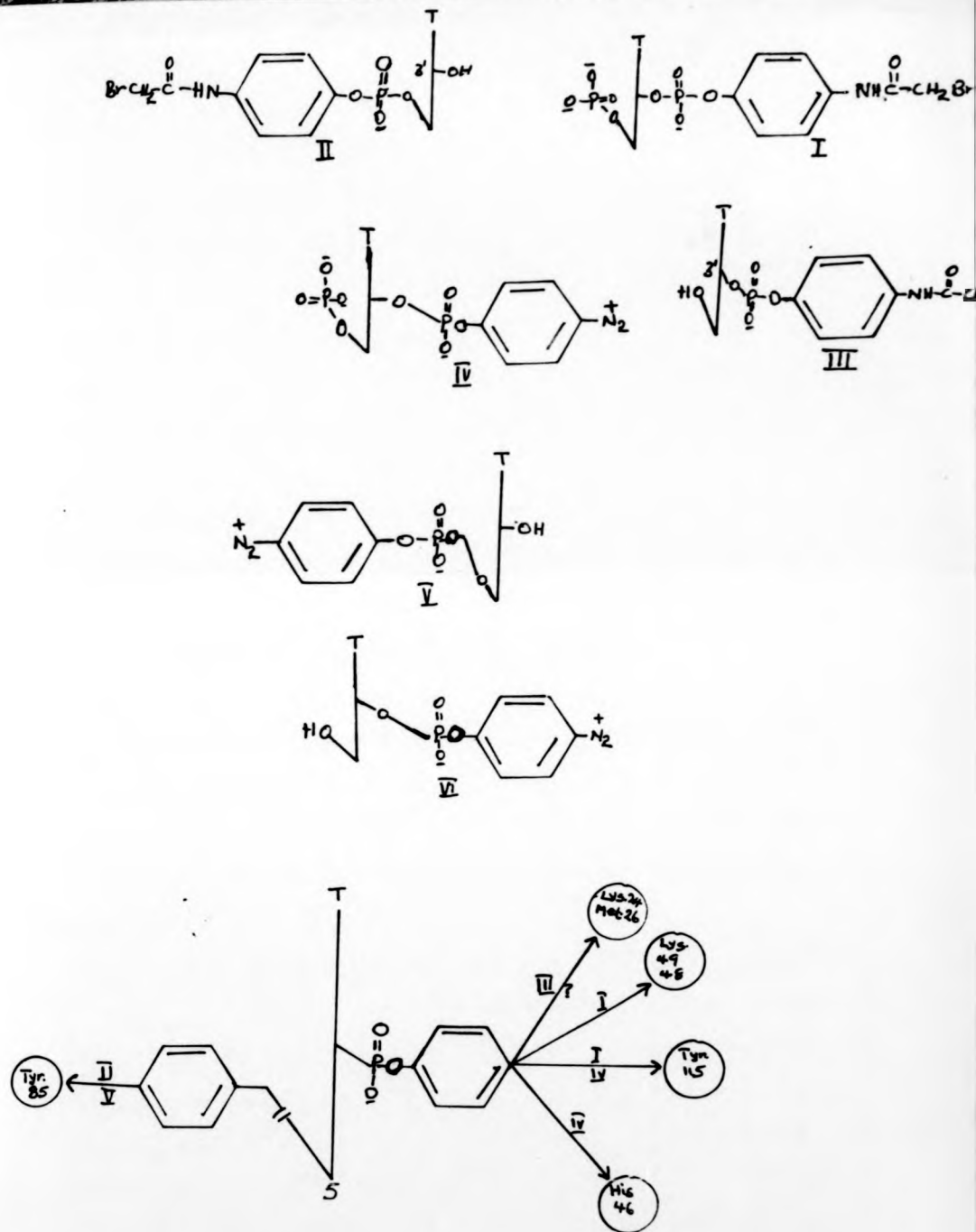


Fig. 3.2 Reagents used for affinity labelling of Staphylococcal nuclease.

R-pdTp-R' lead to release of R-phosphate and dTp-R'. The nature of the R group is not important, but as the present studies show, the nature of the base is important since we have found that the rate of polynucleotide hydrolysis is in the order poly A = poly C > poly G > poly T. However, for a long time it has been known that the nucleoside moiety is necessary since bis- and tris-p-nitrophenyl phosphate esters are not hydrolysed (Anfinsen, et al. 1974).

The enzyme has endo- and exonucleolytic properties similar to other phosphodiesterases, but the basic structural requirements are different.

3.3.3 STEREOCHEMICAL PROBES OF THE ACTIVE SITE

This has also been reviewed by Anfinsen, et al. (1974). The following generalisations can be made. Studies on orientation in the active site are achieved by affinity labelling studies, using bromoacetamido phenyl and diazonium-derivatives of p-aminophenyl nucleotides (Fig. 3.2).

Reagents used for Affinity Labelling of Staphylococcal Nuclease

All these reagents (Fig. 3.2) caused inactivation of the enzyme activity as shown diagrammatically in Fig. 3.2. Specificity of labelling was determined following the stoichiometric inactivation, studying prevention of inactivation, protection experiments, and purification of affinity labelled peptides, etc. Thus, so far the results of various experiments have shown that residues 46-49 are near the substrate binding site and tyr 85 is catalytic in nature, but the crosslinking reagents used to deduce the catalytic function of

tyr 85 may be reacting with another substrate close to the active site. The present study should be able either to prove this or to locate the actual binding and catalytic sites of the enzyme.

Labelling of protein active sites by techniques of affinity labelling has been reviewed (Finn Wold, 1977).

The reagents used fall into the following categories.

k_S reagents - affinity labels designed as direct analogues of the known substrate ligands.

k_{S^+} reagents - transition state analogues - reagents designed to fit reasonable intermediates in activation process through which substrate conversion to product is catalysed.

k^{cat} reagents - (suicide reagents). Similar to the normal enzyme-catalysed conversion of substrate $S \rightarrow P$ through a covalent $E - S$ intermediate, but instead of obligatory enzyme-substrate intermediate k^{cat} reagents here require only an enzyme catalysed activation by which a chemically reactive form of the analogue is produced in situ.

Reaction of this activated species with properly interposed residues in the active site leadsto covalently affinity-labelled derivatives. These are similar to photoaffinity reagents except that in the latter, the production of the active species is by light. Since the specificity of the enzyme is derived from the k^{cat} step and not the k_S step, it is clear that k^{cat} reagents are more specific than the k_S reagents. However, the number of such k^{cat} analogues is few, but those which are known are well used. They require catalytic conversion by the target enzyme, which makes them more specific, since they are chemically unreactive to foreign biomolecules, but have exceedingly reactive

functional groups which can be used in ordinary affinity reagents, since the chemically active inhibitor is produced in situ at active sites. All the above affinity techniques including those previously used for staphylococcal nuclease have multifarious imitations. Some of these have been discussed earlier, and other limitations include:

- (1) Incomplete labelling.
- (2) Breakdown of protection during prelabelling.
- (3) Inactivation unrelated to inhibitor action (e.g. dialysis procedure).
- (4) Exceptional incorporation of the labelled inhibitor during the "specific labelling" phase.
- (5) Random labelling of outside of enzymes causing inactivation.

It is therefore hoped that photoaffinity labelling if used to study staphylococcal nuclease, will provide opportunity to introduce an affinity label which will specifically bind to the active site of the enzyme, and since the reagent z^8 -AMP contains a very reactive residue, this will bind covalently without specificity to the function in the binding site. The achievement of these conflicting requirements - lack of specificity but high reactivity by z^8 -AMP makes it a useful probe for labelling staphylococcal nuclease.

The present study thus aims to synthesize and make chemical use of the analogue of AMP. This will be expected to bind in the nucleotide binding site of the enzyme, but due to lack of a 5'-phosphodiester bond to cleave as substrate cf. 5'-AMP) it is expected to be a competitive

inhibitor. This allows the binding site to be labelled and characterised by identifying the amino acid residues present. The successful demonstration of this technique is intended to serve as a good proof of its potential use in characterisation and elucidation of receptor systems which have so far proved elusive. We also hope to derive novel structural information about the nature of the nucleotide binding sites of the enzyme.

Techniques used include the following.

- (1) Synthesis of z^8 -AMP and 2-(3H)- z^8 -AMP. For z^8 -AMP to be of optimal value, it must be synthesised in radioactive form without loss of the high specific activity of the starting component.
- (2) Labelling of active site.
- (3) Separation of labelled proteins and cyanogen bromide digestion.
- (4) Characterisation and estimation of the labelled peptides.

3.4 MATERIALS AND METHODS

3.4.1 MATERIALS

- (a) (3H)-AMP (16 Ci/mmole) was purchased from Radiochemical Centre, Amersham, U.K.
- (b) Glass-fibre discs were Whatman GF/A (2.5 cm) supplied by Whatman Ltd., Maidstone, Kent, U.K.
- (c) Sodium dodecyl sulphate was a specifically pure reagent for electrophoresis supplied by BDH Chemicals Ltd.,

Poole, Dorset, U.K.

- (d) Nucleotides were products of Boehringer Corporation (London) Ltd., U.K.
- (e) Staphylococcal nuclease (E.C.3.1.47) was obtained from the Boehringer Corporation (London) Ltd., U.K.

3.4.2 GENERAL METHODS

- (a) Gel electrophoresis was performed using a number of different systems:
 - (i) Cylindrical gels were cast in glass tubes at 5% (w/v), 10% (w/v) and 15% (w/v) acrylamide using SDS-phosphate method of Weber and Osborn (1969).

Non-denaturing gels were poured in vertical slabs of 1 mm thickness at a concentration of 5% (w/v) acrylamide for the resolving gel using the high pH discontinuous system of Maizel (1971). The slabs were topped with a 4 cm depth of stacking gel at 3% (w/v) acrylamide.

In all systems the unpolymerised acrylamide was overlaid with n-butanol saturated water and the gels allowed to set. This gives superior interfaces between the resolving and stacking gels compared to water. Samples were prepared as described in the text, and subjected to electrophoresis at constant current (4 mA per gel for cylinders and 20 mA for the slab gel) until the bromophenol blue tracking dye had moved to within 1-2 cm

of the bottom.

- (b) Photolysis experiments were performed with a 100 W high pressure mercury compact arc lamp (Hanovia Lamps Ltd., Slough, Bucks.). The light beam was focussed, by means of a quartz lens (20 cm focal length) on to a 2 mm quartz u.v. cuvette containing the solution to be photolysed. Filtering of the lamp was provided by Pyrex and/or soda glass filters in order to cut out the high energy wavelengths (and produced ultraviolet light above 280-300 nm). The cuvette was kept at a steady temperature by passing over it a stream of constant temperature water (20°) from a peristaltic pump.
- (c) Protein concentration was determined by the method of Lowry, et al. (1951).
- (d) Chromatography was performed by t.l.c. on Merck kieselgel F₂₅₄ plates. Developments were in the following solvent systems:

(A) n-butanol/acetic acid/water	5:2:3 v/v
(B) n-butanol/ .88% NH ₃	1:1 v/v
(C) 2-butanol/formic acid/water	15:3:2 v/v
(D) chloroform/methanol/acetic acid	85:14:1 v/v
(E) methyl acetate/2-propanol/aq. ammonia	9:7:1 v/v
(F) benzene/pyridine/acetic acid	16:4:1 v/v

3.4.3 SYNTHESIS OF Br⁸AMP

This was synthesised in 60% yield essentially by the method of Cartwright and Hutchinson (1980). Spectral characteristics are as follows.

pH 1, λ_{\max} (H ₂ O)	262.5 nm
pH 1, λ_{\min}	234.0 nm
pH 7, λ_{\max}	265.0 nm
pH 7, λ_{\min}	233.5 nm
pH 12, λ_{\max}	265.0 nm
pH 12, λ_{\min}	236.5 nm

Synthesis of z⁸-AMP was also by the method of Cartwright and Hutchinson (1980) in 30% yield. Spectroscopic characteristics are as follows.

$$\lambda_{\max} (\text{H}_2\text{O}) = 282.0$$

$$R_f (\text{solvent A}) = 0.29$$

$$\bar{\nu}_{\max} (\text{Nujol}). 2160 \text{ cm}^{-1}$$

The synthesised z⁸-AMP was converted into its sodium salt, using a Sephadex C50 (Na⁺ form). 2-(³H)-z⁸-AMP was synthesised in an analogous manner, specific activity 9×10^{11} CPM/mole.

3.4.4 ASSAY OF STAPHYLOCOCCAL NUCLEASE

The enzyme was assayed as described by Anfinsen et al. (1974) using poly A as substrate.

Binding of nucleotide to nuclease: The reversible interaction of the nucleotides and the enzyme was studied by a modification of gel filtration technique of Hummel and Dryers (1962). Sephadex G-25

FIGURE 3.3 Lineweaver-Burk plot for the hydrolysis of poly A
by the activity of staphylococcal nuclease in the
absence (○-○-○) or presence (0.0125 M x-x-x
or 0.25 M ●-●-●) of competitive inhibitor Z^BAMP.
(see text)

of poly A
ase in the
-x-x
itor Z^8 AMP.

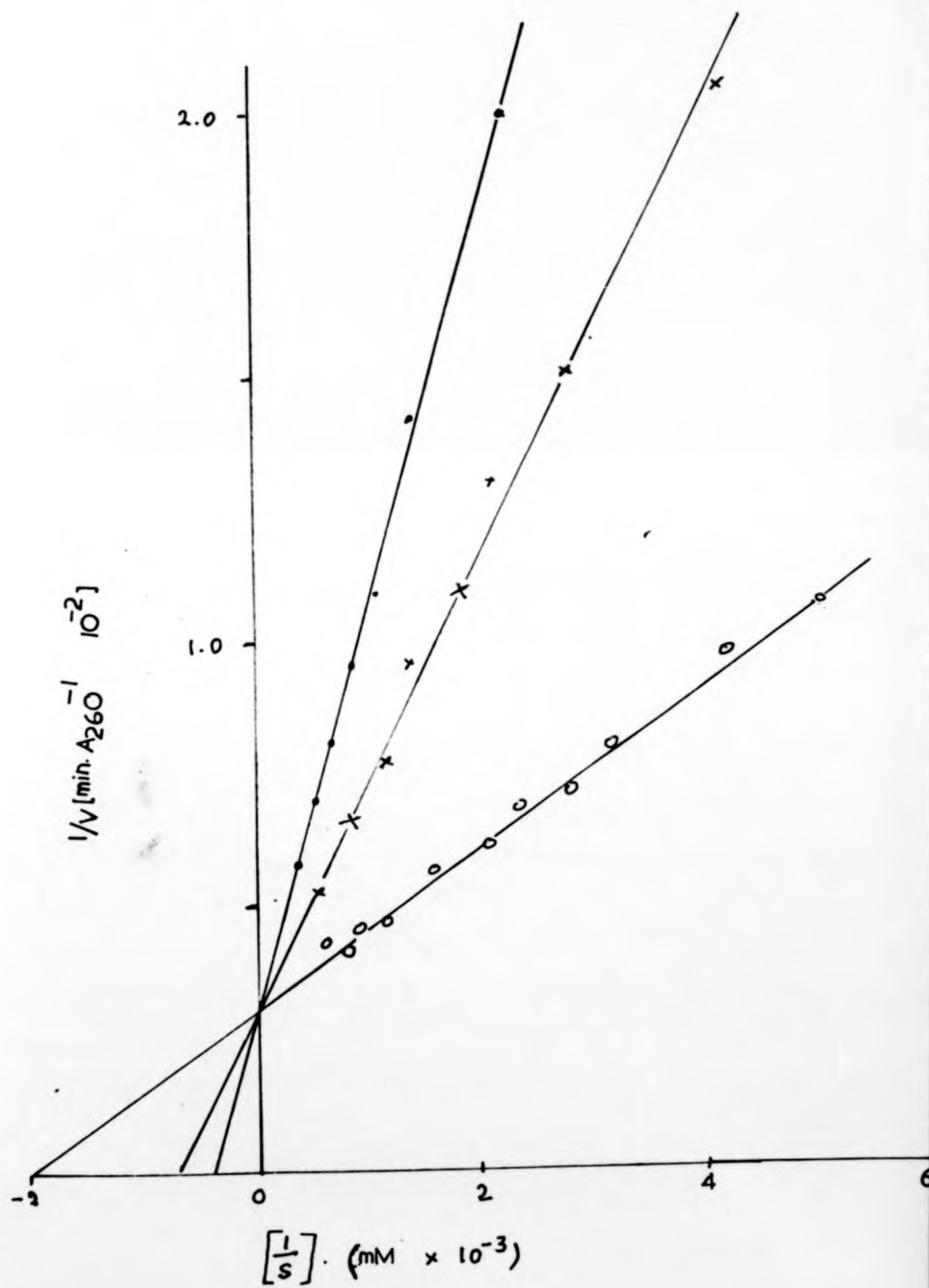


Fig. 3.3

(1.5 x 30 cm) was swollen with 0.1 M borate buffer, pH 8.8, containing 0.1 mM 5'-AMP (37.14 mg/litre). The enzyme was dissolved in the buffer. The nucleotide sample was passed down the column and washed down with 5 ml buffer. 1.2 ml fractions were collected.

The dissociation constants determined were 4.5×10^{-5} M for AMP, literature values for AMP and pTTP were 4.6×10^{-5} M and 5.4×10^{-7} M respectively (Cuatrecasas, et al. 1967).

3.4.5 EFFECT OF z^8 -AMP ON HYDROLYSIS OF POLY(A) BY STAPHYLOCOCCAL NUCLEASE

The hydrolysis of poly(A) by staphylococcal nuclease was studied in presence of z^8 -AMP. The enzyme (11 μ g/ml) was incubated as described, first in the presence of poly(A) of varying concentrations and later in the presence of varying concentrations of z^8 -AMP. The Lineweaver Burk plot is shown in Fig.3.4. The enzyme was also equilibrated with z^8 -AMP in an experiment similar to equilibration analysis as described for 5'-AMP, and the k_i values obtained for both are comparable. The hydrolysis of poly(A) by staphylococcal nuclease was competitively inhibited by z^8 -AMP. A $k_i = 5.5 \times 10^{-4}$ M (mean of 6 determinations) was obtained from a Lineweaver Burk plot. Thus the z^8 -AMP is a good competitive inhibitor of the enzyme. The molar extinction coefficients of the substrate poly(A) and z^8 -AMP to be used in all experiments were determined by the phosphate content method.

3.4.6 STABILITY OF THE STAPHYLOCOCCAL NUCLEASE TO ULTRAVIOLET IRRADIATION

The enzyme (0.1 ml of a solution 11 μ g/ml borate buffer) was

TABLE 3.1

U.V. IRRADIATION OF ENZYME SOLUTION

Photolysis was for 5 min. as described under Methods,
and enzyme used was 11 $\mu\text{g}/\text{ml}$

Addition	U.V.	% Inhibition	
None	-	0.0	0.0
None	+	0.0	0.0
0.5 mM N_3 AMP	-	0.0	0.0
0.5 mM N_3 AMP	+	94.5	93.5
0.5 mM N_3 AMP + 5 mM pdTp	+	17.0	15.0
0.5 mM N_3 AMP			
+			
(i) glycine	-	0.0	0.0
(ii) PBS 50 mM	+	94.0	93.0
(iii) glycine 50 mM	+	93.0	93.5
Double cell experiments	+	95.0	95.0
2-(^3H)- N_3 AMP, 4 mCi/ μmole	+	95.0	95.0

put in a 2 mm quartz cell and exposed to u.v. for various time intervals of 1 to 10 minutes, using a filter to cut off radiation below 300 nm. The irradiated enzyme was taken out and assayed for activity. It was found that the enzyme was stable to u.v. light for a reasonable length of time, 1-10 min. The distance between the lamp and the sample was 6 cm.

3.4.7 PHOTOAFFINITY LABELLING EXPERIMENTS

Solutions of staphylococcal nuclease (1.1 μg in 0.1 ml) and $z^8\text{-AMP}$ (5.5×10^{-3} M, 0.1 ml) in 0.1 M sodium borate pH 8.8 were irradiated as described above. An opaque screen was interposed between the lamp and cuvette after 30 sec., 1 min., etc. and the solution was assayed for enzymic activity. The enzyme was inactivated after 5 min., and to determine the rate of inactivation of the nuclease activity experiments as shown above were set up and photolysis was performed at intervals of 30 sec. for 5 min. The

rate of inactivation was little altered when 5.5×10^{-4} or 5.5×10^{-5} M $z^8\text{-AMP}$ were used. Similarly, the rate of inactivation was not altered when either 10^{-4} , 10^{-3} , 10^{-2} M glycine or 4-aminobenzoic acid were added separately to the borate buffer before irradiation. When pdTp (5×10^{-5} M) was added to the $z^8\text{-AMP}$ little inactivation of the nuclease had occurred after 5 min. irradiation. If thymidine (5×10^{-5} M) was added to the reaction mixture in place of pdTp, inactivation of the nuclease occurred after 5 min. irradiation. When the $z^8\text{-AMP}$ was irradiated for 5 min. before addition to the nuclease, complete retention

of enzyme activity was observed even after further irradiation for 10 min.

3.4.8 PROTECTION EXPERIMENTS

Thymidine 3'-5'-diphosphate tetrasodium (pdTp) was used. The enzyme (11 μ g) 0.1 ml (10^{-4} M) of z^8 -AMP and 0.1 ml (10^{-4} M) pdTp were incubated and photolysed for 1,2,3,4 or 5 min. The samples were then assayed for enzyme activity using poly(A) as before. The blank consists of all the above solutions except that the mixture was not photolysed but it was kept in the dark for a corresponding period of time.

The experiments were repeated, changing the concentration of pdTp used. The results are shown in Table 3.1. Similar experiments were carried out as described above with various concentrations of z^8 -AMP. It was found that the most effective ligand concentration is 5.0×10^{-4} M to 5.0×10^{-5} M, the former being near the concentration of the actual inhibitor constant of the ligand z^8 -AMP.

3.4.9 NITRENE SCAVENGING EXPERIMENTS

The following mixtures were incubated and photolysed for 5 min.: 0.1 ml (11 μ g/ml) enzyme, 0.1 ml (10^{-4} M) z^8 -AMP. The blank consists of the enzyme and z^8 -AMP without photolysis. In another experiment, solutions 10^{-4} M, 10^{-3} M, 10^{-2} M of scavengers (4-aminobenzoic acid, or glycine) were prepared, and photolysed for 5 min. in the presence of 0.1 ml (10^{-4} M) z^8 -AMP. The blank consisted of the components above not photolysed, but assayed as for the normal experiments. The inactivation of the enzyme proceeded

at the same rate as that described earlier.

A double cell experiment was also performed, and it was found that the effects observed for the PBS and glycine solutions are not due to absorption of radiation by both compounds.

3.4.10 PHOTOAFFINITY LABELLING WITH 2-(³H)-z⁸-AMP

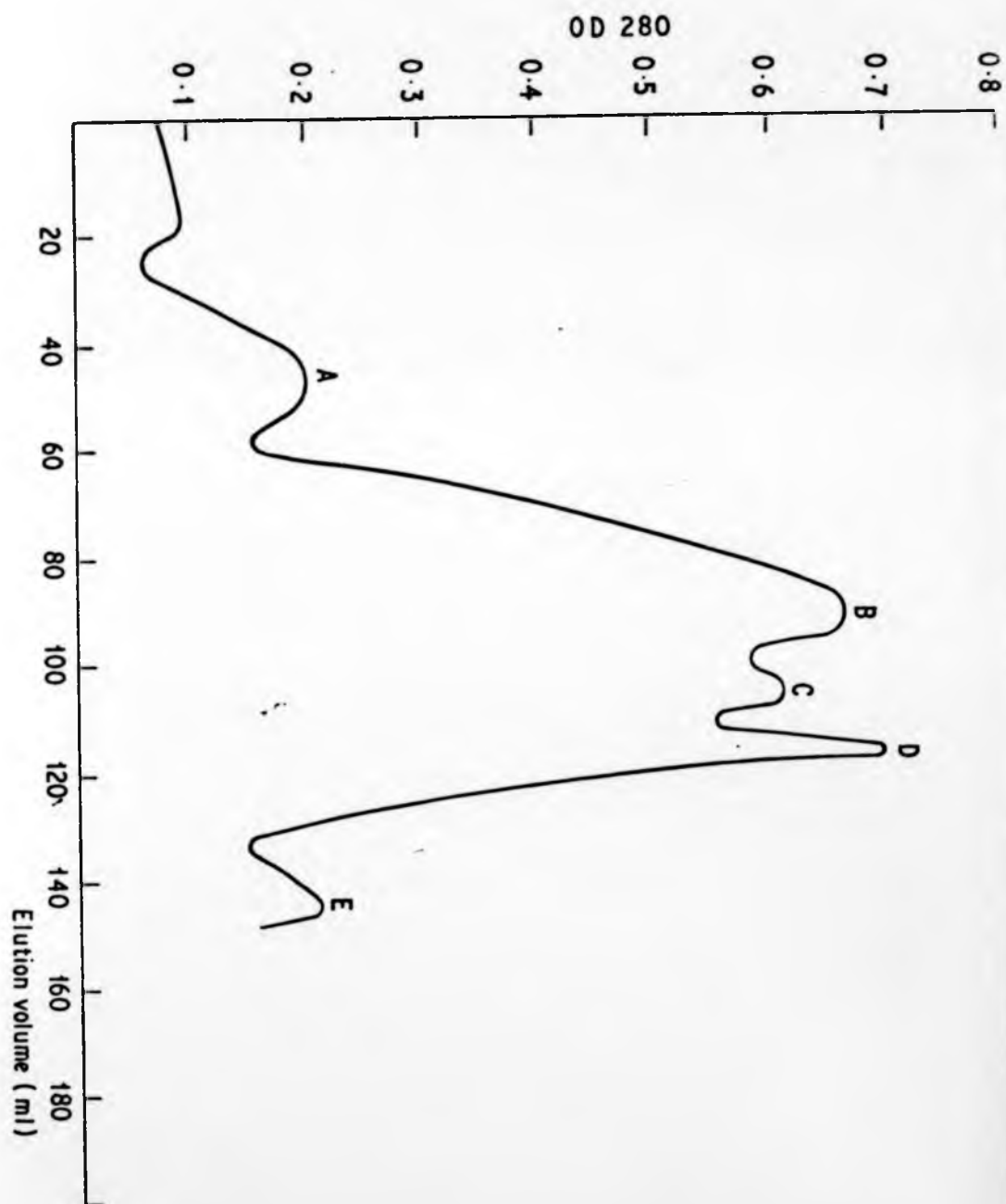
Photoaffinity labelling with 2-(³H)-z⁸-AMP (5.5 x 10⁻³ M, specific activity 9 x 10¹¹ CPM/μmole) was used in the irradiation experiments described above and after a given time the reaction mixture was applied to a Sephadex G-25 column (1 x 100 cm) which was then washed with 0.1 M sodium borate buffer, pH 8.8. These chromatographic conditions completely separated unreacted z⁸-AMP from the enzyme. When excess 2-(³H)-z⁸-AMP (46,000 CPM) was introduced into the column with the enzyme in the same solution without irradiation, no appreciable counts were obtained in the first 40 ml eluates, even though fractions collected contained the protein peak. However, after 40 ml was collected, > 95% of the nucleotide radioactivity was eluted.

The experiments involving photolysed inactivation of the enzyme using 2-(³H)-z⁸-AMP were repeated as described for z⁸-AMP. (Table 3.2).

Location of Site of Labelling

After the photocatalysed inactivation of the enzyme with 2-(³H)z⁸-AMP, followed by removal of unreacted 2-(³H)-z⁸-AMP by gel chromatography 100 μg of the protein was subjected to CNBr digestion as described by Taniuchi and Anfinsen (1966), using 30 μmolar excess of CNBr (82 mg). The protein was dissolved in 70% formic acid to make a 1% total solution, and cyanogen bromide (82 mg) was added to the sample and kept at 25°C for 20 hours. Distilled water (0.2 ml) was added, and the solution was lyophilised to remove excess CNBr.

FIGURE 3.4 CNBr Peptides from radioactively photo affinity
Labelled S. Nuclease. Elution from Sephadex G-50
(See text.)



y photo affinity
from Sephadex G-50

TABLE 3.2

INCORPORATION OF (³H)-z⁸-AMP INTO STAPHYLOCOCCAL NUCLEASE
AFTER IRRADIATION

Time (min.)	Mole (³ H)-z ⁸ -AMP Incorporated per mole nuclease
0	0
1	0.1
2	0.35
3	0.55
4	0.70
5	0.95
10	0.95
5*	0.07

*In presence of 10⁻² M pdTp.

The results are averages of 2 determinations.

TABLE 3.3

N-TERMINAL ANALYSIS OF CNBr DIGESTED PEPTIDES

DNP-amino acid derivatives were employed for identification

	<u>No. of moles Expected</u>	<u>No. of moles Observed</u>	<u>Peak (Fig. 3.4)</u>
DNP-valine	2	2.1	C and D
DNP-Thr	1	1	E
DNP-Ala	1	1	B
DNP-tyr	1	2	A

DNP-tyr was the only one out of line; number of moles corresponds to what was expected for other DNP-amino acids present in the protein

Chromatography of the digest on Sephadex G-50 column (1 x 200 cm) using 0.02 M acetic acid containing 0.2% NH_4OH for elution gave poorly resolved peptides (A-E, Fig. 3.4). Other chromatographic systems (e.g. Dowex 50, Sephadex G-25 or DEAE cellulose) were tried unsuccessfully in attempts to improve the resolution of the protein peaks. The peak containing the first fraction is a mixture of peptides and was discarded (see N-terminal analysis later). Fractions collected are 2 ml, and elution rate 10 ml/hour. Fractions are assayed using absorbance at 280 nm. Peaks C and E contained 20% and 80% of the total radioactivity applied to the column. The peak fractions of C and E were collected and their peptide contents determined as 6 μg and 8.4 μg respectively.

Fluorescence technique, using fluorescamine, provided an accurate and convenient assay for quantitating the actual amounts of peptides bearing the radioactive label.

In one set of experiments, the labelled enzyme was digested with CNBr, separated on Sephadex G-50, the radioactive peptides C and E were subjected to complete amino acid analysis. In another set of experiments, the labelled enzyme was digested as described above, separated on Sephadex G-50, and the peaks obtained (A,B,C,D,E) were subjected to N-terminal amino acid determinations using the DNP-amino acid derivative (see Table 3.3).

3.4.11 PEPTIDE MAP EXPERIMENTS

Experiments were done using dansyl chloride. These were carried out as described by Schemer (1967), and the chromatography was performed using solvents C and E, and E and F. The Schemer solvents gave lower intensity, probably due to the presence of pyridine.

3.4.12 ELECTROPHORESIS EXPERIMENTS

Various electrophoresis experiments were performed as described before, for the following purposes.

- (1) Purity of the nuclease -SDS- gel electrophoresis.
- (2) Peptides hydrolysed by CNBr - attempts were made to resolve these by slab-gel electrophoresis and fluorescamine coupled-disc gel electrophoresis.
- (3) Dansyl coupled disc-gel electrophoresis.
- (4) To resolve the hydrolysed CNBr peptides:
 - (i) Slab-gel electrophoresis (Kabayo, J. P. Personal Communication)
 - (ii) Dansyl derivatives coupled to disc-gel electrophoresis (Fisher and Press, 1974)
 - (iii) Fluorescamine coupled to disc-gel electrophoresis (Singh et al. 1962)
 - (iv) As (ii) and (iii) with slab-gel electrophoresis (O'Farrell 1975).

The modifications of the original work of Roseblatt, et al. (1975), gave the best result of all the techniques attempted.

- (5) Because (2) and (3) did not give good results, alternative modification of the straightforward SDS-gel electrophoresis was also performed. This gave better results.

3.5 RESULTS AND DISCUSSION

3.5.1 BROMINATION, AZIDATION AND PURIFICATION OF NUCLEOTIDES

The synthesis proceeded well and the yields were reasonably good, being 60% (br⁸AMP) and 40% (z⁸-AMP) respectively. Problems encountered were in isolation processes. Compensation has to be made for the high buffer (0.6 M) concentration required to maintain effective pH 4.5 and the need to avoid loading the ion exchange column with excessively concentrated salt solutions. Thus, pH 4.5 was maintained at 0.5 M potassium acetate buffer. Lee and Kaplan (1975) have described a simple procedure for preparation of br⁸AMP in high yield involving chloroform extraction of unreacted bromine and lyophilisation of the aqueous phase. However, it was not useful in the present study, since only column purified products would be suitable for the introduction of the azido group under anhydrous conditions. Previous studies on the introduction of the azido group onto the 8-position of the adenine base utilised sodium azide in DMF as the nucleophile (Muneyama, *et al.* 1971). However, the commercial product, tetramethylguanidinium azide (Papa, 1966), which was more soluble in organic solvents, gave consistent substitution of bromine in good yield. Concerning the same reaction, a variety of DMF-soluble azides have been described, e.g. tributylammonium azide (Koberstein, *et al.* 1976) and hydrazoic acid in triethylamine (Schäfer, *et al.* 1976). These reagents are in no way better than tetramethylguanidinium azide. The synthesis of z⁸-AMP by the displacement of bromine by azide in br⁸AMP was

Floure 3.5

Change in ultra-violet spectrum of z^8 AMP on photolysis in water. Spectra were recorded with the pyrex filter in place at

**(a) 0" (b) 30" (c) 60" (d) 90" (e) 120" and
(f) 150" at a concentration of 4.45×10^{-4} M**

photolysis in
filter in place

) 120" and

A

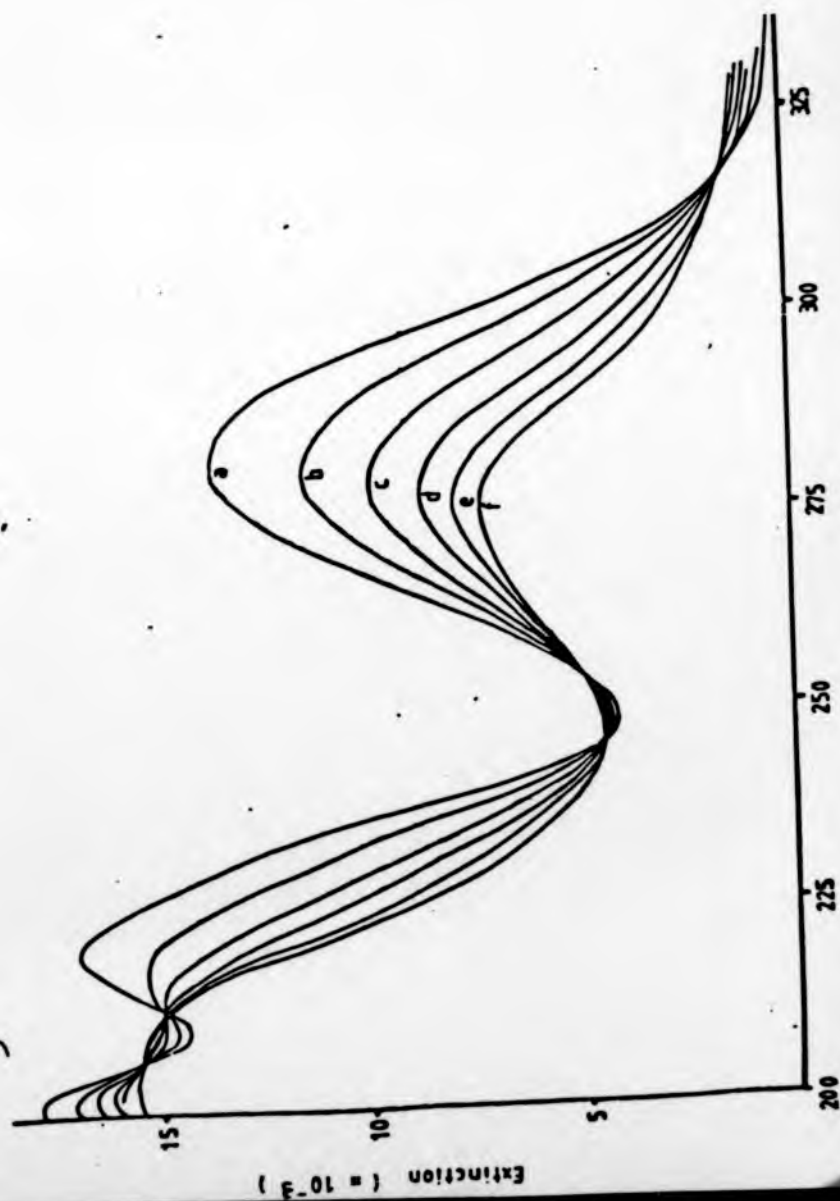
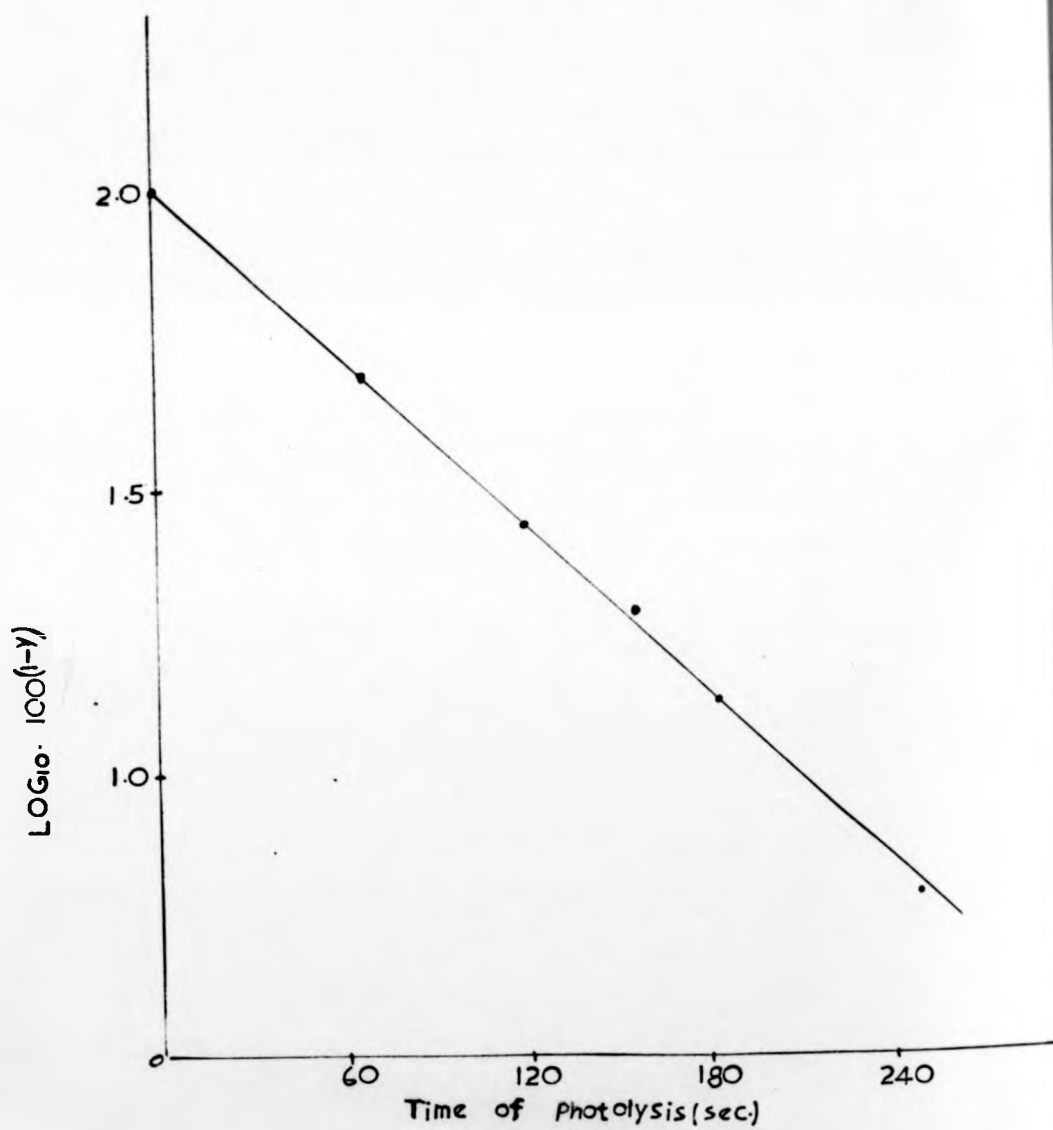


FIGURE 3.6 First order kinetic plot for z^8 -AMP photolysis. Conditions are described in text.

The first order rate constant $k = 1.05 \times 10^{-2} \text{ s}^{-1}$ and $t_{1/2} = 63 \text{ secs}$. The quantity $(1-y)$ where y = extent of reaction ($0 \leq y < 1$) is similar to $(a-x)$ and is derived from O.D. data at 270 nm.



accomplished by using higher concentration of the tetramethyl guanidinium azide than hitherto employed in some earlier reports. The azido nucleotide synthesised, however, has the same characteristics as reported in earlier works.

The z^8 -AMP showed strong infra-red absorption around 2160 cm^{-1} , a region characteristic of asymmetric stretching for covalent azides (Lieber, et al. 1966)

3.5.2 PHOTOLYSIS OF z^8 -AMP

The photolysis of z^8 -AMP in water proceeded rapidly and smoothly, as disclosed by the change in u.v. spectra with time, and the number of *isosbestic* points obtained (Fig. 3.5). Under the conditions employed here (Pyrex glass filter, 100 W lamps), the half-life of photolysis derived from a first-order rate plot is 64.0 seconds (Fig. 3.6). These results are similar to those observed by Koberstein, et al. (1976).

The product of photolysing the z^8 -nucleotides was thought to be 8-hydroxyl amino-AMP formed by insertion of the putative nitrene into water. As evidence, it was observed that the product of reaction between br^8 AMP and hydroxylamine displayed a u.v. spectrum very close to that observed for the final photolysis product of z^8 -AMP. It was also observed that the photolysis of z^8 -AMP in water leads to other products which are deep red and remain at the origin on silica t.l.c. It is likely that 8,8'-azoAMP was a major complex product since the attack of aryl nitrene on precursor azide to yield azo compounds

is a common reaction (Reiser and Wagner, 1971). Fig. 3.7 shows the probable major reaction products of the photolysis.

The z^8 -AMP was irradiated at 20° as shown in the experimental section. After irradiation the wavelength changes shows the λ_{\max} shifted from 281.5-275 nm, and no other changes seem to occur. A change in optical density was also noticed to have occurred with a decrease during photolysis.

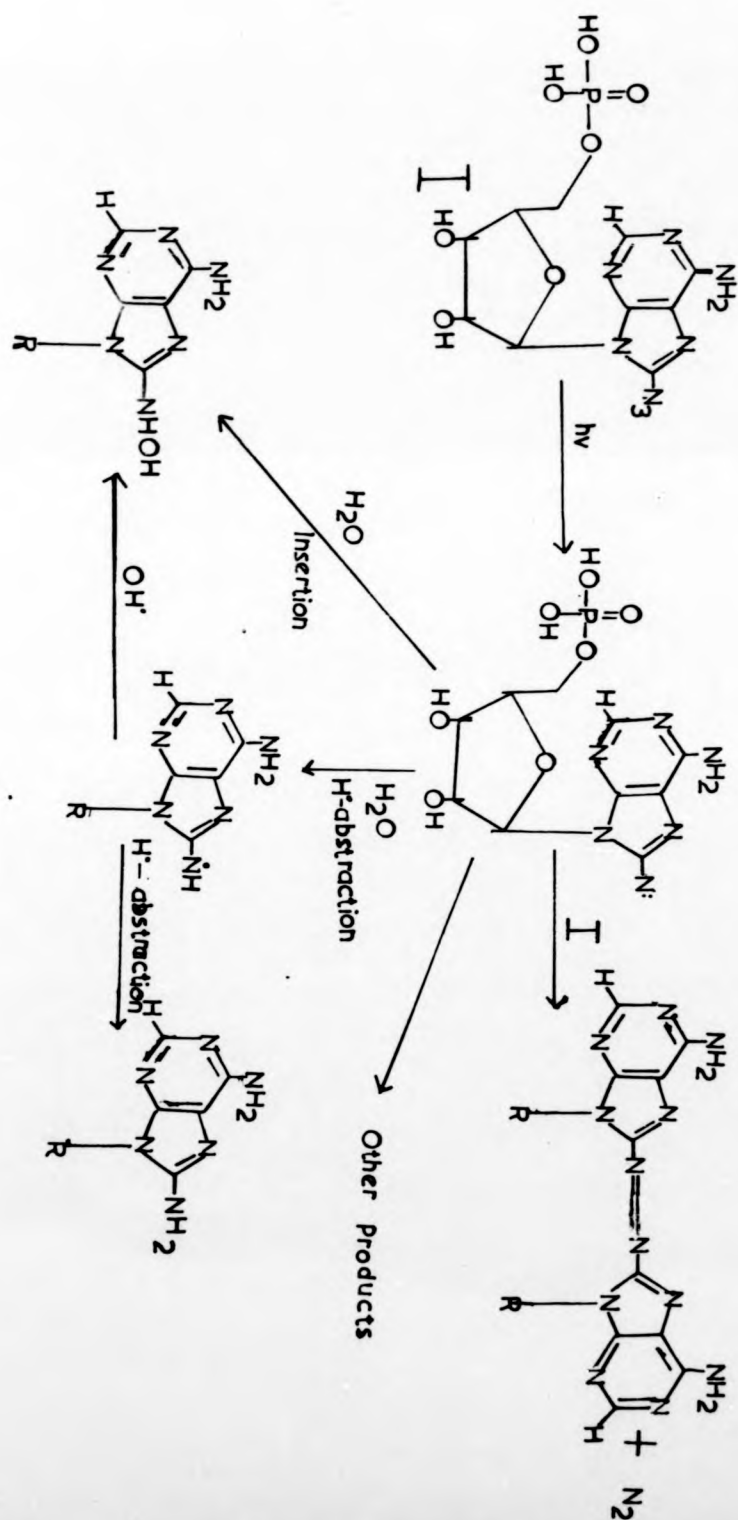
Double reciprocal Lineweaver Burk plot shown was obtained by measuring the velocity after incubation against concentration of polynucleotide, and the value of k_m was calculated as 1.6×10^{-3} M. Values of k_i observed by Lineweaver Burk plot and gel filtration techniques were 0.55×10^{-3} M and 0.95×10^{-3} M respectively.

It is interesting to compare this value with the k_i for AMP on the same enzyme 0.046×10^{-3} M (see next section). This change in k_i value (factor 10) may be due to the fact that the synthesised z^8 -AMP may be in the syn conformation. The AMP has anti-conformation, thus this is a reflection of the specificity of the enzyme, although the enzyme still recognises the syn conformation of the z^8 -AMP derivative as its competitive inhibitor reasonably well, if less readily than AMP.

The size of the azido moiety may also be relevant here. The azido group is generally linear and studies of covalent azides have shown that the N-N bond is 1.5 \AA by molecular orbital calculations. The C-N bond in azobenzene is 1.45 \AA (Treinin, 1970). Thus, the azide group may extend in a linear way to a distance of 3.82 \AA from the purine nucleus. The $\overset{\alpha}{C}-\overset{\beta}{N}-N$ bond angle is usually 120° , but dipole moment studies using p-chloro and p-bromophenylazides show

FIGURE 3.7 Reaction products in the photolysis of Z^8 AMP
under aqueous conditions.

R-β-D-ribose-5'-monophosphate



that the dipoles nearly cancel each other, and the bond angle is thus assumed to be much wider. This is probably because the α -nitrogen possesses lone-pairs of electrons in p-orbitals and can almost certainly interact by electron-delocalisation with the aromatic ring, thus causing a bond angle closer to 180° . In the aromatic azido-nucleotide, this means the bulky azido group is probably not able to bend away from severe non-bonded interaction with substituents of the furanose ring, with the result that relief of steric overcrowding can be achieved most effectively by adoption of the syn conformation, i.e. this conformation may relieve the steric overcrowding which resulted from the interaction of the azido group and 2'-H of the sugar ring.

3.5.3 PHOTOAFFINITY LABELLING OF STAPHYLOCOCCAL NUCLEASE USING z^8 -AMP

As mentioned above, z^8 -AMP acts as a competitive inhibitor of the substrate poly A when the enzyme was tested in the presence of these two substrates. This has a consequence in this experiment in as much as it means that irradiation experiments described can safely be assumed to result in binding of the photoaffinity label z^8 -AMP to the nucleotide binding site of the enzyme and not to any other site. The k_i was calculated as 0.55 mM. Because this value is lower than the k_m for poly A, it shows a tight binding to the nucleotide binding site, and that the k_i is a true equilibrium constant while k_m is not.

z^8 -AMP is an efficient photoaffinity label for staphylococcal nuclease as the enzyme is rapidly inactivated when it is irradiated in the presence of dilute solutions of this nucleotide. The rate of

loss of enzyme activity is unaltered if the irradiations are carried out in the presence of nitrene scavengers such as glycine or 4-aminobenzoic acid indicating that true photoaffinity labelling is taking place. The reactive nitrene generated on photolysis of z^8 -AMP appears to interact with the active site of the nuclease, as pdTp, a strong inhibitor of the nuclease can protect the enzyme from photoactivated deactivation by z^8 -AMP. Solutions of thymidine, which does not bind to the nuclease, do not protect the enzyme from photoactivated deactivation by z^8 -AMP confirming the hypothesis that the pdTp is protecting the enzyme active site. In addition, kinetics of the binding show that z^8 -AMP is a competitive inhibitor of poly A, and photolysis of the nucleotide in the presence of the enzyme produces a colourless solution (irradiation of the nucleotide alone produces orange-red colour) and loss of over 90% activity of the enzyme. This must be due to specific photoaffinity labelling in the active site.

3.5.4 PHOTOAFFINITY LABELLING OF STAPHYLOCOCCAL NUCLEASE USING 2-(3 H)- z^8 -AMP

When 2-(3 H)- z^8 -AMP is used in the photolysis experiments, one molecule of tritiated nucleotide is covalently bound to each nuclease molecule after 5 min. and the amount of binding does not increase significantly on prolonged irradiation.

When 2-(3 H)- z^8 -AMP and pdTp are irradiated in the presence of the nuclease, virtually no tritium is incorporated into the protein after 5 min., again confirming that the nitrene generated from z^8 -AMP

TABLE 3.4

PARTIAL AMINO ACID ANALYSIS OF PEAKS C AND E
OBTAINED AFTER CNBr DIGESTION OF LABELLED
STAPHYLOCOCCAL NUCLEASE

Residue	Peak C Found (n moles)	99-149 Expected (n moles)	Peak E Found (n moles)	33-65 Expected (n moles)
Ala	5.4	6	1.8	2
His	1.2	1	1.1	1
Leu	4.2	5	2.6	3
Phe	0	0	2.0	2
Val	3.3	4	2.6	2

AMINO ACID ANALYSIS OF THE RADIOACTIVE PEPTIDES WAS

PERFORMED BY :

MACROMOLECULAR ANALYSIS SERVICE

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P.O.BOX 363 BIRMINGHAM.

interacted with the active site of the nuclease. The radioactively labelled nucleases obtained in the experiments described above can be degraded by cyanogen bromide, and chromatography of the degradation mixture on Sephadex G-50 gives five poorly resolved peptide peaks (A-E), one of which (E) contained 80% and another (C) 20% of the total radioactivity (fluorimetric assay, radioactive labelling, peptide maps (see Section 3.4.11)).

The peak fractions of peptides C and E were subjected to amino acid analysis after hydrolysis with hydrochloric acid. From these analyses it was clear that peptide E corresponds to residues 33-65 and peptide C to residues 99-149 of the staphylococcal nuclease (Table 3.4). Comparison by polyacrylamide gel electrophoresis of the CNBr digest of the native nuclease and tritiated nuclease obtained as described above, indicates that two peptides in the tritiated nuclease have different mobilities. N-terminal and complete amino acid analysis using DNP-derivatives confirmed the identities of these two peptides.

The formation of two tritium-labelled peptides following cyanogen bromide degradation of the tritiated nuclease implies that the nitrene produced on photolysis of z^8 -AMP has a comparatively long lifetime. This would allow the nitrene to react with several amino acid residues at the active site, and such behaviour has been observed for the nitrene generated from p-azidocinnamoyl chymotrypsin (Bridges and Knowles, 1974) and with a carbene generated from pUp (Havron and Sperlin, 1977). It is also relevant that electrophilic affinity labelling of the active site of staphylococcal nuclease can result in the labelling

of amino acid residues in either or both cyanogen bromide peptides C or E. For example, the diazonium derivative of deoxythymidine 3'-p-amino aminophenyl) 5'-phosphate reacts specifically with tyrosine 115, while the diazonium derivative of deoxythymidine 3¹-(p-amino-phenyl)phosphate reacts with both tryptophan 140 and histidine 46 (Cuatrecasas, 1970). This indicates that peptides C and E contain amino acid residues in the vicinity of the active site as is confirmed by both x-ray crystallographic studies and by partial degradation of the nuclease (Anfinsen, et al. 1974).

Also, γ -³²P-AMP can function as a photoaffinity label under mild conditions when little cell death occurs (Cartwright, I. L. unpublished observation) and hence polymers derived from this nucleotide could be used for the photoaffinity labelling of cell membranes and other complex systems.

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